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LOYOLA UNIVERSITY CHICAGO

SELECTIVE EXPANSION OF B CELLS BY INTESTINAL MICROBIOTA

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
MASTER OF SCIENCES

PROGRAM IN MICROBIOLOGY AND IMMUNOLOGY

BY
KARINA OCHOA
CHICAGO, IL
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LIST OF ABBREVIATIONS

Ab	Antibody
Ag	Antigen
AID	Activation-induced deaminase
BCR	B cell receptor
BLAST	Basic Local Alignment Search Tool
BM	Bone marrow
C	Constant region
CDR	Complementary-determining region
CHO	Chinese hamster ovary cell
D	Diversity region
DC	Dendritic cell
DSM	Difco sporulation medium
DTT	Dithiothreitol
Fab	Fragment antigen-binding
FACS	Fluorescence-activated cell sorting
FAE	Follicular associated epithelium
FBS	Fetal bovine serum

FDC	Follicular dendritic cells
FR	Framework region
GALT	Gut-associated lymphoid tissue
gDNA	Genomic DNA
H	Heavy chain
HSC	Haematopoietic stem cell
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
IgL	Immunoglobulin light chain
J	Joining region
L	Light chain
LB	Luria broth
LP	Lamina propia
M	Membrane cells
MHC	Major histocompatibility complex
mLN	Mesenteric lymph node
OD	Optical density
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PLB	Passive lysis buffer

PP	Peyer's patches
RAG-1/2	Recombination activating gene 1 and 2
SAg	Super antigen
SpA	<i>Staphylococcus aureus</i> protein A
SR	Sacculus rotundus
TBST	Tris buffered saline and Tween 20
TCR	T cell receptor
TLR	Toll-like receptor
V	Variable region
WT	Wildtype

ABSTRACT

In rabbits, the majority of the antibody diversity is generated in gut-associated lymphoid tissues (GALT) where B cells migrate after leaving the bone marrow (BM). B cells that seed GALT expand polyclonally, and the immunoglobulin (Ig) genes of essentially all B cells undergo somatic diversification leading to an expanded primary antibody repertoire. Somatic diversification of the Ig genes occurs by somatic hypermutation and also by somatic gene conversion. These processes occur in an antigen- and T cell-independent mechanism. The development and expansion of the antibody repertoire requires interaction of GALT with intestinal microbiota.

Serum Ig secreted from the B cells of normal rabbits shows a predominant $V_{H\alpha}$ allotype over the V_{Hn} allotype. In contrast to this, *ali/ali* mutant rabbits (*Alicia*), which contain two mutant V_{Ha}^2 alleles, show at birth a prevalence of the V_{Hn} allotype. Upon contact with the gut microbiota, the *ali/ali* rabbit V_{Hn} B cell population stops proliferating and in turn, cells with $V_{H\alpha}$ allotype start expanding. These events do not occur by gene replacement or by secondary Ig gene rearrangement on the unexpressed Ig allele. It is possible however, that the expansion of $V_{H\alpha}$ cell takes place by selective expansion induced by the intestinal microbiota.

Bacteria promote B cell proliferation and the formation of B cell follicles. In addition, rabbits raised under germ-free conditions have under-developed GALT and

poor antibody responses to antigenic challenges. The molecular mechanism by which bacteria promote GALT development and function remains unclear.

For my thesis I investigated the mechanism by which commensals drive GALT reactions, particularly the processes that lead to the activation and migration of B cells in GALT. The Knight Lab model suggests that B cells that enter GALT follow a chemokine gradient to the follicular associated epithelium (FAE), where they may encounter bacteria. These events presumably provide the B cells with signals that may lead to their activation and relocation to other regions of the follicle. B cells can be activated in a T cell independent stimulation by B cell receptor (BCR) engagement, or through Toll-like receptor (TLR) signaling. I hypothesized that surface molecules of selected bacterial species in the gut of rabbits cause the activation and expansion of B cells.

To determine if commensals bind surface Ig I isolated bacteria from the intestines of rabbits, and found that commensal can bind to Ig, independently of their specificity. Particularly, I detected a ~20kDa bacterial molecule from at least three species that immunoprecipitated with FvIg. To determine whether bacteria provide signals for activation and migration, I stimulated B cells with bacterial molecules and detected the expression of B7, which is expressed on activated B cells but not on resting B cells. I found that bacterial molecules from commensals provide signals that induce the activation of B cells. In addition, I stimulated cells through BCR or through TLR2, and

found an increase in the expression of chemokine receptors (CCR6, CXCR5, CCR7 and CXCR4), known to be expressed on B cells as they move throughout the follicle.

The evidence collected in these studies suggests that bacterial molecules can bind to B cells via surface Ig. This supports the idea that in the FAE bacteria provide B cells with signals that may lead to their activation.

These studies provide insights into the mechanism by which interactions between the host and intestinal microbiota alter the B cell repertoire in an antigen independent manner. My findings contribute to our current model of B cell activation and migration in GALT.

CHAPTER ONE

LITERATURE REVIEW

ANTIBODY DIVERSITY

Theories of Antibody Diversity

With the discovery of antibodies (Ab) in the last decade of the 1800s, the scientific community became interested in the mechanism that leads to antibody diversity. In 1897 P. Ehrlich published his “Selectionistic Theory”, which stated that Ab producing cells expressed a large variety of receptors on the cell surface, each able to interact with a different antigen (Ag). Ehrlich proposed that these preformed Abs can associate with a particular toxin or Ag "like a key in a lock“, causing stimulation for the production of more Ab to released to into the blood (Brand and Roth, 2008). However, this theory could not explain how Abs are developed against practically any molecule in nature, and was later dismissed.

A few decades later the “Instructionistic Theory” was explained by L. Pauling and F. Haurowitz. This theory stated that the Ab protein was a template molecule that could be folded into specific shapes by the interaction/instruction with Ag. Once folded into a specific shape, the Ab would subsequently interact only with that particular Ag. This hypothesis could not explain affinity maturation, self/nonself discrimination, how Ab

continues to be produced in the absence of Ag, or why secondary responses are much more rapid and robust than the initial immune response (Brand and Roth, 2008).

The “Clonal Selection Theory” was proposed a few years later by F. M. Burnet, D. W. Talmage and J. Lederberg. This theory stated that each Ab-producing cell bears, through a random process, Abs of a single molecular species unique for a specific Ag. According to this postulate, when an Ag binds its specific Ab, it induces both the clonal expansion of Ab-producing cells, and the creation of a population of memory cells (Llewelyn, 1992; Hood, 2008). This theory could not explain how a limited number of genes could provide an infinite number of Ag specificities (Brand and Roth, 2008).

Around the same time, W. J. Dreyer and J. C. Bennett revolutionized the Immunology field with their “Germline Theory” for Ab diversity, which stated that many genes existed to encode each Ab (Gearhart, 2006). By then, it was known that Abs are composed of a variable region (V) and a constant region (C), but it was not known whether one gene or more was involved in the expression of the whole protein. The finding that each antibody had its own amino acid sequence in the variable region suggested a separate gene for each antibody chain produced. They proposed that each class of C region is encoded by one gene, but that large numbers of genes exist for the V domains (V_H and V_L). In addition, they correctly suggested that antibody genes would be formed by recombination between these genes during B cell development (Llewelyn, 1992).

Five years later, in 1970, T. T. Wu and E. A. Kabat successfully mapped and aligned all of the amino acid sequences of the Abs, determining their variability. Using these sequences, they created a metric of variability at each amino acid residue position, and plotted this variability across the 107 residues of the light chain V region. This diversity figure led them to identify extreme hypervariable regions: the Ag-binding or complementarity-determining region (CDR) of the Ab light (L) chain. This led them to the proposal of the “Somatic Theory” for antibody diversity. They suggested that the CDRs were encoded by episomes that were able to recombine with a few germline genes, consequently creating a somatic mechanism for generating Ab diversity (Hood, 2008).

The controversy over how Ab diversity occurs was put to an end with the publication by S. Tonegawa and N. Hozumi, in 1976. They showed indisputable data that provided evidence for the rearrangement of Ig genes. Their approach was to compare DNA from Balb/c mouse early embryos with that from plasmacytoma cells (differentiated cells). By nucleic acid hybridization experiments they found that the pattern of embryo DNA showed two components, one of which hybridized with *C*-gene sequences and the other with *V*-gene sequences. The pattern of the tumor DNA showed a single component that hybridized with both *V*-gene and *C*-gene sequences, and that it was smaller than either of the components in embryo DNA (Hozumi and Tonegawa, 1976). Their results was the first direct evidence that showed that separate genes encode the V and C regions of Abs, and that genes are somatically rearranged during B-cell differentiation (Brand and Roth, 2008).

In the years that followed, the *J* (joining) segment of both L and heavy (H) chains was identified. In 1980 P. Early and L. Hood identified the *D* (diversity) segment of $V_H(D)J_H$ by isolating myeloma clones and sequencing the V and J regions (independently), and comparing them with the sequence of a rearranged V_H gene encoding the complete heavy chain. They found that there were non-coding sequences that include conserved nucleotides in both the V and the J regions. Moreover, they observed in the rearranged V_H gene solely, the presence of nucleotides encoding a few extra amino acids. They proposed that these nucleotides originated from a third germline gene segment, which they named “D” for diversity (Early, 1980).

Several other mechanisms that are involved in the antibody diversity were identified over the past three decades. These include the activation-induced deaminase (AID), which initiates hypermutation by modifying Ig genes (Gearhart, 2006); and the recombination activating gene 1/2 (*RAG-1/2*), actively involved in gene recombination (Brand and Roth, 2008).

Every theory formulated over the years to explain the mechanism of Ab diversity was partially correct. Each of them contributed to the modern understanding of how huge numbers of Ags are recognized by Abs. We now know that the recombination of hundreds of gene segments, somatic hypermutation and affinity maturation are combined to generate Ab diversity. This remarkable process is completed by the natural mechanism of selection.

Site of antibody diversification in rabbits

In humans and mice, combinatorial joining of multiple V , D , and J gene segments results in a vast array of $V(D)J$ genes that form the primary Ab repertoire (Vajdy, 1998). However, other species like chicken, sheep and rabbit, which utilize a very limited number of V_H and V_L genes in the $V(D)J$ gene rearrangements, use a different strategy to increase their poor combinatorial mechanism. Their primary antibody repertoire is diversified post-rearrangement by either somatic gene conversion or somatic hypermutation, or both (Pinheiro, 2011). In the case of rabbits, the immunoglobulin heavy chain (IgH) locus contains approximately 100 V_H gene segments; however, V_{HI} (3'-most V_H gene) is preferentially used in the $V(D)J$ gene rearrangements in most B cells (Knight and Becker, 1990).

The diversification of the Ab repertoire in chickens occurs in the bursa of Fabricius shortly after hatching, and for sheep it occurs in the ileal Peyer's patches after birth; in rabbits this process occurs in the appendix. This was first suggested by Cooper (1968) after performing neonatal appendectomies and finding that these rabbits had reduced numbers of circulating lymphocytes and immunoglobulins. This was later confirmed when rearranged V_H region genes of B cells isolated from the appendix of 6 weeks old rabbits were sequenced. The results demonstrated that the primary antibody repertoire occurs early in rabbit B cell development within the appendix germinal centers, and that it may occur by gene conversion and somatic hypermutation (Knight and Crane, 1994; Weinstein, 1994).

THE RABBIT AS A MODEL ORGANISM

Historical perspective of the rabbit as a model organism

As an animal model, the rabbit has historically been used in a variety of biomedical research areas, toxicology studies, and for research involving the immune system (Püschel, 2010). To this day, the rabbit is still widely used in research, primarily because of the high quality and quantity of its antibodies. Studies of the rabbit immune system have greatly contributed to our knowledge of the structure, function and regulation of antibodies (Pinheiro, *et al.*, 2011). This organism has also been used in all types of basic science studies, including nutrition, reproduction, and embryology (Craig, 2012).

Rabbits originated in the Iberian Peninsula around 2000 B.C. Some attempts at domestication were made during the Greek and Roman periods however, true domestication was not initiated until about the 16th century. The first scattered reports of the use of rabbits as laboratory animals began to appear by the middle of the 19th century (Püschel, 2010; Pinheiro, *et al.*, 2011).

The rabbit family includes 10 genera and 25 species. The European rabbit (*Oryctolagus cuniculus*) diverged into two subspecies, *O. cuniculus algirus* and *O. cuniculus cuniculus*, approximately 1.8 million years ago. The worldwide migration of humans over the centuries has mediated the dispersal of this species. We can now find the European rabbit in Continental Europe, England, Australia, New Zealand, North and South America, and North Africa (Pinheiro, *et al.*, 2011).

Allotypic divergence of rabbit antibodies

In rabbits, B cell development occurs in bone marrow (BM), and nearly all VDJ gene rearrangements utilize the same V_H gene, V_{H1} . This factor limits the extent to which combinatorial joining of multiple V genes contributes to antibody diversity (Knight and Becker, 1990).

The unique feature of Rabbit Ig allotypes set them apart from mice, humans and other mammals. In rabbit, the gene locus controlling the V region of the IgH chain shows extensive allelic diversity. The V_H regions of rabbit antibodies can display different allotypic motifs: a , x , y or z . Serological surveys of domestic rabbits defined three “ a -positive” allotypic lineages: $a1$, $a2$, and $a3$ (Pinheiro, *et al.* 2011). The divergence between these allotypes is of approximately 20% amino acid sequence differences. The allelic specificities of $a1$ and $a2$ are correlated with several amino acid differences in framework regions (FR) 1 (Tonnel, *et al.*, 1983).

These allotypes are mostly encoded by the predominantly rearranged gene, V_{H1} . Serum Ig of rabbits typically reacts with anti- V_{Ha1} , anti- V_{Ha2} , or anti- V_{Ha3} allotypic antibodies. However, 10 to 20% of serum Ig does not react with these antibodies. This is referred to as V_{Hn} (V_{Ha} -negative) Ig, which lacks V_{Ha} allotype-specific determinants (Horng, 1976; Zhu, 1999; Esteves, 2004). V_{Hn} Ig is encoded predominantly by V_{Hx} , V_{Hy} , and V_{Hz} genes, which reside over 50 kb upstream of the V_{H1} gene (Zhu, 1999).

About 80 to 90% of circulating Ig molecules are derived from the V_{H1} gene and express the V_{Ha} allotypic markers (Margolies, 1977). The V_H regions of the remaining

10 to 20% of Ig molecules are encoded by the V_{Hn} genes (Roux, 1981). The molecular basis for preferential usage of the V_{H1} gene in VDJ rearrangements remains unanswered.

Alicia rabbit (ali/ali)

In 1986 Kelus and Weiss established a variant strain that originated from a heterozygous a^1/a^2 male. These rabbits have a variant V_{Ha2} allotype-encoding allele, *ali*, which has a 10-kb deletion of genomic DNA encompassing V_{H1} (Becker and Knight, 1990; Allegrucci, *et al.*, 1990). In contrast with wildtype rabbits, nearly all Ig in young *ali/ali* rabbits (designated *Alicia*) are V_{Hn} .

Newborn *Alicia* rabbits possess a predominant V_{Hn} B cell population, but as these rabbits age, the number of V_{Ha} B cells increases, becoming the predominant allotype (Pospisil and Mage, 1998; Zhu, 1999). Mutant *ali/ali* rabbits lack the IgH V_H gene segment, V_{H1} . Analysis of nucleotide sequences of the promoter region showed that more than 80% of the VDJ rearrangements in older *Alicia* rabbits utilize either the functional V_{H4} or V_{H7} genes localized upstream of V_{H1} . The V_{H4} and the V_{H7} genes have 7 (out of 11) specific nucleotides associated with the allotype $a2$ (Zhu, 1999; Sehgal, *et al.*, 1998).

The V_{H4} gene encodes molecules known as V_{Ha} . However, the V_{Ha} - μ heavy chains encoded by V_{H4} do not pair with surrogate light chain in the BM to form a functional preBCR. Consequently, most V_{Ha} B-lineage cells do not pass the preB cell checkpoint during B cell development and are deleted in the BM (Zhu, 1999). As a result, cells utilizing V_{Hn} genes become the dominant B cell type that successfully

develops in BM and exits to the periphery. These V_{Hn} B cells comprise the vast majority of B cells in gut-associated lymphoid tissues (GALT) and in the periphery for the first 6 weeks of life (Pospisil, 1995). This dominance of V_{Hn} B cells changes dramatically between 6 and 11 weeks of age, when the few V_{Ha} (V_{H4} -utilizing) B cells that complete development in the BM exit to the periphery, expand rapidly and become the dominant B cell type.

Rhee, *et al.* (2004) demonstrated that this phenomenon does not arise from V_H gene replacement or from secondary Ig gene rearrangements on the unexpressed Ig allele, as initially suggested. They also demonstrated that this shift occurs in GALT and requires intestinal microbiota. Their results suggest that a B cell superantigen may promote the positive selection of V_{Ha} B cells in GALT, by stimulating B cells in an antigen-nonspecific, polyclonal manner (Rhee, *et al.*, 2004).

B-CELL SUPERANTIGENS

Superantigens (SAGs) are bacterial molecules capable of triggering innate and adaptive immune responses. SAGs were initially thought to interact exclusively with T cells, and accordingly, their association to T lymphocytes has been extensively described. In contrast to conventional antigens, SAGs do not require to be processed into peptides, or even presented by major histocompatibility complex (MHC) molecules. Instead, the intact SAG molecule binds outside the MHC groove and interacts directly with the T-cell receptor (TCR) (Zouali, 1995).

Extensive studies conducted in recent decades have concluded that SAgS are not exclusive to T cells but have specificity for B cells as well. B cell superantigens are molecules that bind to surface Ig outside of the conventional Ag binding site. These bacterial molecules can stimulate B cell differentiation and Ig secretion. It has been found that these molecules bind to conserved FR amino acid residues in the V region of the heavy or light chains (V_H or V_L , respectively) (Severson, 2010).

Staphylococcus aureus protein A (SpA) is one such B cell superantigen. The interactions of SpA with the fragment antigen-binding (Fab) region of membrane Igs can stimulate a large fraction of B cells, contributing to lymphocyte polyclonal expansion. The binding specificity is restricted to the Fab heavy chain V_H3 family in humans and other mammalian species. In the Fab, the residues in contact with SpA are located in the V_H region framework β -strands and the inter-strand loops most remote from the antigen combining site (Graille, *et al.*, 2000).

Rhee, *et al.* (2004) compared amino acid sequences of rabbit V_{Hn} and V_{Ha} Ig, and identified a putative V_H ligand binding site in the FR1 and FR3 regions for a bacterial B cell superantigen. This study demonstrated that these binding sites are clustered on the external face of the V_H domain with their side chains exposed for potential interaction with SAgS.

Superantigens are molecules that bind outside the conventional antigen binding site. Since almost all B cells in rabbit utilize the same V_H gene (V_{HI}) during VDJ gene rearrangements, it is possible that a bacterial superantigen interact with all B cells, independent of their BCR specificity (Knight and Winstead, 1997).

B CELL DEVELOPMENT

In mammals, B cells are generated from haematopoietic stem cells (HSCs). They develop in the BM before they migrate into the blood to reach peripheral lymphoid organs. Reticular cells, which surround BM sinuses, have been shown to be associated with B cells, making them likely candidates for providing the factors required for B-cell development. Both *in vitro* and *in vivo* studies indicate that osteoblasts are also essential regulators of the development of B cells in the bone marrow. The mechanisms that regulate the association between developing B cells and their cellular niches, including microenvironmental factors, remain unclear (Nagasawa, 2006).

B cell precursors are divided in fractions A (pre-pro B cells), B (pro-B cells), C (pro-B cells) and D (pre-B cells). This classification was arranged according to their differential expression of a range of cell-surface markers during development in the bone marrow. Immature B cells, which are generated from fraction D cells, exit the bone marrow and reach the spleen, where they mature into peripheral mature B cells and subsequently into plasma cells (Nagasawa, 2006). Plasma cells, which develop following the activation of mature B cells by Ag in peripheral lymphoid organs, return and colonize the BM. Most plasma cells, present in constant numbers in the BM, are long-lived and not derived from the differentiation of proliferating, activated B cells (Manz, 1997).

During their development and differentiation, B cells undergo immunoglobulin-gene rearrangement. B cell precursors first assemble a heavy-chain DJ rearrangement, which is then followed by heavy-chain variable (V)DJ gene rearrangements (Nagasawa,

2006). In humans and mice, combinatorial joining of multiple V, D, and J gene segments results in a vast array of V(D)J genes that form the primary Ab repertoire.

B cells that undergo combinatorial joining leave the bone marrow to seed the periphery. This Ab repertoire is expanded further by somatic mutation after Ag stimulation (Griffiths, 1984). The combinatorial joining of species like chicken (IgH and IgL) and sheep (IgL) occurs by a limited number of *V*, *D*, and *J* gene segments followed by somatic diversification of the *V(D)J* genes in an exogenous Ag-independent manner (Reynaud, 1987; Reynaud, 1991). These immature B cells are now expressing a functional BCR, which provides them with antigen specificity. Cells that successfully cross this phase enter the periphery as transitional B cells.

The ability of the adaptive immune system to provide protection against pathogens requires a diverse BCR repertoire that can recognize a broad range of foreign molecules. Diversity is generated early in development by random rearrangement of immunoglobulin genes, and by somatic hypermutation (Cambier, 2007).

In rabbits, most B cell development occurs in the BM early in ontogeny, decreasing dramatically within a few months after birth. During their development in the BM, the diversification of the B cell antibody specificities is limited. Knight and Becker (1990) demonstrated that nearly all *VDJ* gene rearrangements utilize the same V_H gene, V_{HI} , significantly limiting their antibody diversity. Instead, the majority of the antibody diversity is generated in GALT between 3 and 8 weeks of age, where B cells migrate after leaving the BM (Vajdy, 1998). The B cell expansion and the somatic diversification of Ig genes occur by a gene conversion-like mechanism and by somatic hypermutation

(Becker and Knight, 1990; Lanning and Knight, 1997). This process occurs in germinal center-like structures in an antigen- and T cell-independent mechanism, as demonstrated by Pospisil and Mage (1998). This differs from germinal center reactions in mice which are dependent on antigenic stimulation. Other studies have shown that this process of somatic diversification requires interaction of GALT with intestinal microbiota (Rhee, *et al.*, 2004).

GUT-ASSOCIATED LYMPHOID TISSUE (GALT)

The gut associated lymphoid tissue, is the largest collection of lymphoid tissues located in the intestines of some mammals. GALT is composed of mesenteric lymph nodes (mLNs) and Peyer's patches (PP), and B and T lymphocytes in the intestinal lamina propria (LP) and epithelium (Forchielli, 2005).

A Roman physician, Rufus of Ephesus, identified mLNs for the first time during the second and first centuries BC. The PPs of the small intestine were discovered centuries later by the Swiss anatomist Johann Conrad Peyer. The existence of PPs in the cecum and colon of a number of small mammals was not reported until 1884, by the Irish zoologist George Edward Dobson (Eberl and Lochner, 2009).

GALT is overlaid with specialized areas named follicle-associated epithelium (FAE), which are involved in sampling and transporting antigens from the lumen of the intestines to the mucosa. The FAE contains a varying number of thin membrane (M)

cells with a unique ability to translocate antigens and a variety of bacteria into the mucosa of the intestines (Yamanaka, 2003).

B cell expansion in GALT

It is well established that intestinal bacteria are essential for the normal structural and functional development of the mucosal immune system. Studies on germfree mice demonstrated that these exhibit smaller PPs and fewer intraepithelial lymphocytes, compared to germfree mice colonized with single or multiple species of bacteria (Forchielli, 2005). A recent study using germfree rats demonstrated in immunohistochemistry experiments, that the FAE contains only a few T and B cells. It was also observed that the B cells of these germfree rats were negative for B7, a co-stimulatory molecule necessary for T cell activation. After bacterial colonization, activated B cells achieved a spatial position in the FAE facilitating their encounter with luminal Ags (Yamanaka, *et al.* 2003).

In rabbits, the appendix, sacculus rotundus (SR), and PP comprise the GALT, where B cells migrate after leaving the BM (Crane, 1996). To determine whether GALT is necessary for generating the primary Ab repertoire in rabbits, Vajdy (1998) surgically removed the organized GALT shortly after birth, and compared the antibody repertoire to that of normal rabbits. This study showed by nucleotide sequence analysis that GALT, in particular the appendix, is essential for the somatic diversification of IgM *VDJ* genes of peripheral B cells. Additionally, the authors found diminishing numbers of peripheral B cells after removal of the GALT. These findings also indicate that GALT functions as a

primary lymphoid organ for the post-natal expansion of B cells. The rabbit appendix is thought to function as a secondary lymphoid organ in adult rabbits (Weinstein, *et al.*, 1994b).

Later studies demonstrated that the intestinal microbiota, are necessary and required for the diversification of the Ab repertoire, and B cell expansion in the rabbit. This was first demonstrated by Lanning (2000) while examining VDJ gene diversification of rabbits in which the appendix was ligated at birth to prevent microbial colonization. By 12 weeks of age nearly 90% of the Ig VDJ genes were undiversified, indicating that the intestinal microflora is required for somatically diversifying the Ab repertoire. A similar arrest in B cell expansion and appendix follicle development is also observed in new born rabbits with a ligated cecum (Hanson and Lanning, 2008). Colonization of intestinal microbiota begins at birth. The maternal microbiota can be a source of bacteria colonizing the newborn's intestine. Colonization can also be affected by environmental factors and by infant feeding patterns (Forchielli, 2005).

Hanson and Lanning (2008) demonstrated by *in situ* hybridization that B cells enter GALT 2 days after birth and migrate to the FAE. Here, the cells presumably receive signals from bacteria located in pockets of M cells located along the epithelium. In addition to this, B cells may be stimulated by one or more of several mechanisms, including CD40L and Toll-like receptors (TLRs) (Yeramilli, *et al.*, 2011). The stimulated B cells proliferate and upregulate activation-induced cytosine deaminase (AID); and in the basolateral region of the follicle, the Ig genes undergo somatic diversification.

In rabbits, the development and expansion of the antibody repertoire are dependent on interactions between GALT and the intestinal microbiota. Additionally, in the appendix, SR and PP, bacteria promote B cell proliferation and the formation of B cell follicles. However, not all bacterial species equally induce GALT development, as demonstrated by Rhee, *et al.* (2004) when using different combinations of bacteria into germfree appendices. In this study the combination of two bacterial species, *Bacillus subtilis* and *Bacillus fragilis*, was sufficient to stimulate B cell development in rabbit GALT. Finally, rabbits raised under germ-free conditions have under-developed GALT and poor antibody responses to antigenic challenges (Rhee, *et al.*, 2004). To this date the molecular mechanism by which bacteria promote GALT development and function remains unclear.

To investigate the mechanism by which bacteria promote B cell development in GALT, K. Severson (2010) tested whether bacterial derived superantigen-like molecules could mediate B cell proliferation in GALT. The authors of this study identified and described a superantigen-like protein, ExsK, found on the surface of bacterial spores. Their results suggested that surface proteins on bacterial spores may polyclonally stimulate B cells and promote their development in GALT.

B CELL ACTIVATION

Resting and activated B cells display distinct phenotypes and functional properties. Resting B cells are ineffectual whereas activated B cells are capable of triggering T cell activation (Ding and Shevach, 1996). In addition, activated B cells can

differentiate to form either plasma cells capable of antibody secretion, or memory cells that provide long-lived protection against secondary infection (Harwood and Batista, 2010).

B cell activation can be initiated by the ligation of the BCR with antigen, which results in the production of protective antibodies against potentially pathogenic invaders. However, the specific mechanism by which antigen engagement triggers activation processes is not yet completely understood.

The binding of specific antigen to the membrane Ig component of the BCR initiates intracellular signaling and results in B cell activation (Harwood and Batista, 2010). The dimerization of monomeric BCR on the surface of B cells, upon stimulation with antigen, is indicative of B cell activation. Immunofluorescence microscopy experiments demonstrate that immunoglobulins on the surface of B cells can spontaneously segregate to specific areas. In these areas the Ig molecules crosslink with each other and form caps (Schreiner, 1976).

Other receptors involved in the activation of B cells include members of the Toll-like receptor (TLR) family, initially found in *Drosophila melanogaster*. Human homologues to these transmembrane receptors were first described in by R. Medzhitov (1997). This study demonstrates that TLRs induce the activation of NF-kappaB and the expression of NF-kappaB-transcription factors. These events induce the expression of inflammatory cytokines IL-1, IL-6 and IL-8, as well as the expression of the co-stimulatory molecule B7. Subsequently identified mammalian homologues were found to recognize a series of conserved microbial products referred to as pathogen-associated

molecular patterns (PAMPs) (Leadbetter, *et al.* 2002). Some of these products are lipopolysaccharide (recognized by TLR4), microbial lipoproteins and peptidoglycans (recognized by TLR2), and CpG DNA (recognized by TLR9) (Forchielli, 2005).

To determine whether B cell activation and antibody responses require TLR signaling, Pasare and Medzhitov (2005) performed B cell transfer experiments. In this study purified B cells from wildtype, MyD88 knockout, TLR4 knockout and CD40 knockout mice were transferred into mice that lack mature B cells, followed by immunization with lipopolysaccharide (LPS). MyD88 is a universal adapter protein as it is used by most TLRs to activate the transcription factor NF-kappaB, while CD40 is expressed on the surface of B cells and it is essential in cellular activation. The results of this experiment suggested that the formation of germinal centers and the percentage of germinal center B cells were diminished in mice that received TLR4 knockout or MyD88 knockout B cells. In addition to this, the activation of transcription factors involved in B cell activation was significantly impaired in mice that receive these knockout cells. The authors concluded that TLRs expressed on B cells have a direct role in B-cell activation.

Studies have shown that synchronized signaling through BCR and TLRs augments expression of NF-kappaB, and enhances B cell activation, proliferation and differentiation. Other costimulatory molecules involved in the activation of B cells are CD40 and B7. As mentioned before, CD40 assists in the activation, proliferation, differentiation, survival and generation of memory B cells (Jain, 2011).

To test if B cell activation can occur independently of BCR, S. Jain (2011) stimulated resting B cells through TLR2 and CD40, both molecules crucial for innate and

adaptive immunity. The results of this study demonstrated that the combination of stimuli through these two receptors augmented the activation, proliferation and differentiation of B cells.

The up-regulation of expression of the B7 family of molecules has been considered to be the primary reason for this functional conversion of activated B cells. Using different stimuli, Ding and Shevach, (1996) demonstrated that activated B cells expressed comparable levels of many of the known counter-receptors for costimulation and intercellular adhesion (B7-1, 87-2, HSA, ICAM-1). They also demonstrated that activation of B cells via CD40, and to a lesser extent with LPS, induced potent B7 expression. This was confirmed by Yang and Wilson (1996), who administrated CD40L-deficient mice with an activating antibody to CD40. This experiment resulted in the increased expression of B7 on spleen cells.

Thus far, the evidence collected in different studies indicates a correlation in innate and adaptive immune components that results in modulating the functions of B cells. To take it one step further, S. Jain (2013) asked if the simultaneous stimulation of TLR2 and costimulatory molecule, B7 results in the activation of resting B cells. As a result, it was found that these B cells exhibited a significant level of activation and proliferation. The study concluded that signaling of TLRs in conjunction with costimulatory molecules help in strengthen humoral immune responses.

B CELL MIGRATION VIA A CHEMOKINE GRADIENT

The working model for B cell development in the Knight Lab proposes that resting B cells leave the BM approximately 2 days after birth and enter GALT. Once in GALT the cells follow a chemokine gradient to the FAE, where the chemokine CCL20 (ligand to CCR6) is expressed, presumably by epithelial cells. To this day it has not been verified if a specific process occurs to B cells in the FAE. However, it is believed that in this region B cells may encounter bacteria that pass through M cells. Pape (2007) demonstrated by tracking a fluorescent antigen that small antigen can diffuse into the follicle, where is later acquired by resting B cells. This process occurs without dendritic cell (DC) or T cell help. However, it is also possible that DCs located in the FAE capture bacterial molecules from the lumen and ‘show’ them to B cells. These events presumably provide the B cells with signals that may lead to their activation and relocation to other regions of the follicle.

According to the Knight Lab working model, the B cells expressing CXCR5 migrate next to the follicular dendritic cell (FDC) zone, where stromal cells and FDCs express the chemokine CXCL13 (Gunn, 1998). After receiving additional stimulatory signals from FDCs, and possibly also from bacteria, the cells migrate to the T cell areas, attracted to CCL21, a chemokine secreted by stromal cells which is also the ligand for CCR7 (Legler, 1998). Finally, the B cells migrate to the basolateral end of the follicular area where CXCL12, the ligand for CXCR4, is expressed. Here the cells proliferate and the Ig genes undergo somatic diversification. This model is relevant to understanding the

mechanism by which bacteria from the intestines of rabbits provide signals for the activation and migration of B cells in GALT.

Chemokines are small secreted proteins that serve as critical extracellular mediators of cell migration, particularly in the immune system. These chemoattractants can mediate selective recruitment of particular cell types evident in certain tissues (Comerford and McColl, 2011). Inflammatory chemokines are expressed by circulating leukocytes and other cells only upon activation, whereas homeostatic chemokines are constitutively expressed (Allen, 2007).

CONCLUSION

The current working model of B cell development in rabbit was derived mainly from studies designed to understanding the mechanism and site(s) for B cell development and activation, and the generation of the antibody repertoire. Although it has been demonstrated that bacteria plays a central role in the above mentioned processes as well as in germinal center formation, the specific mechanism is not yet known.

For many years the Knight Lab has focused on experiments designed to identify the bacterial molecule(s) that drives the activation and migration of B cells in the GALT of rabbits. In this thesis document I will give details about the strategies I used to gain answers to some of these questions.

CHAPTER TWO

MATERIALS AND METHODS

Bacteria sorting by fluorescence-activated cell sorting (FACS)

One 4 week old rabbit from the colony maintained by K. L. Knight at Loyola University Chicago, was sacrificed and its appendix dissected. The contents were centrifuged to remove debris and fecal material. The supernatant optical density (OD_{600nm}) was measured to estimate the concentration of bacteria ($OD_{600nm} = 0.985$). The supernatant was stained in the cold for 1 hour with 10 μ g/mL biotinylated a⁺FvIg, biotinylated a⁻FvIg, and biotinylated Fc γ (negative control). After washing, the cells were stained with 1 μ g/mL APC conjugated to Streptavidin. Bacterial cells were sorted by FACS Aria cell sorter, located at the FACS Facility, Loyola University.

16sRNA sequencing

Bacterial isolates from the intestine of rabbits were grown in 5% sheep blood agar plates. One colony was picked, re-suspended in 5mL Luria broth (LB) and grown by agitation for 4 hours at 37°C. Genomic DNA (gDNA) was extracted as follows: 1mL of each bacterial sample was centrifuged at 4000rpm for 5 minutes, and the pellets re-

suspended in 0.85% NaCl solution. The samples were centrifuged again, re-suspended in 1mL 1X TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA), and treated with 10 μ L Proteinase K (10mg/mL). The samples were frozen for 1 hour at -80°C; digested for 1 hour at 60°C; and boiled for 15 minutes at 95°C. A volume of 0.5mL phenol-chloroform (1:1) was next added to the tubes containing 0.5mL of the bacterial DNA solutions, and mixed gently for 5 minutes. The tubes were centrifuged at 10,000rpm for 10 minutes at room temperature, and the aqueous phase transferred into new labeled tubes. Next, 3M NaOAc (1/10th of total volume) and 100% EtOH (3 volumes) were added to each tube. The tubes were placed at -80°C for 1 hour, and then centrifuged at 13,000rpm for 15minutes. The pellet was air-dried and finally re-suspended in 20 μ L dH₂O.

An internal segment of the 16sRNA region, size 1.5kb, was amplified from the gDNA by Polymerase chain reaction (PCR) using internal primers P6 and P7 (Table 1), and run on a 40% polyacrylamide gel to make sure the correct gene segment was amplified. The samples containing the correct amplified gene were run in a 1% agarose gel. The 1.5kb DNA bands containing the amplified 16sRNA region were cut out of the gel, placed in clean eppendorf tubes and solubilized in 6M NaI. The tubes were incubate at 68°C for 10 minutes or until the agarose was melted. The DNA was run through a Wizard PCR resin column with a filter, and then washed with 6mL 80% isopropanol. The DNA solution collected in the filter was centrifuged at 12,000rpm for 2min, the filter placed in a clean tube, and the DNA fragment eluted with 20 μ L of dH₂O.

The 1.5kb DNA segment was ligated into the pGMT-EZ (2746bp) vector and incubated overnight at 16°C. Competent *E. coli* cells (DH10B) were then transformed with the vector containing the 1.5kb insert using electroporation (Cell Porator®, Gibco BRL). The cells were plated on LB-Ampicillin plates coated with 20µL IPTG and 50µL X-Gal for blue/white screening, and incubated at 37°C for 18 hours.

The plasmid extraction was performed by Super Quick assay as follows: white colonies were picked from each plate and grown in 5mL LB-Ampicillin for 18 hours at 37°C. Microcentrifuge tubes were loaded with 200µL of the cultures and centrifuged at 12,500rpm for 3minutes. The pellet was re-suspended in 50µL 10% Lysozyme; boiled for 3 minutes and spun again for 10 minutes. The supernatants were transferred into clean tubes and 40µL isopropanol was added. This mixture was centrifuged at 12,500rpm for 30minutes at 4°C, and the pellet was left to air-dry. The insert was digested from the pGMT-EZ vector with the restriction enzyme EcoRI. The digested samples were loaded onto 1% agarose gels; run for 1 hour; and visualized to identify the samples that contained the correct size vector and insert.

A Wizard® Miniprep was used to purify the DNA from the correct samples. This was done by centrifuging the remainder of the culture of each sample at 3,000rpm for 10min. The pellets were re-suspended in 400µL of re-suspension solution, followed by 400µL of lysis solution and shaken immediately. After 5 minutes, 400µL of neutralization solution was added and the tubes were agitated immediately. This mixture was next spun two times at 3,000rpm for 10 and 5 minutes, and the supernatant transferred into clean tubes. The supernatants were run through a Wizard resin column

with a filter, and the DNA solution collected in the filters was centrifuged at 12,000rpm for 2min. The filters were placed on a clean tube, and the DNA fragment eluted with 100 μ L of dH₂O. The purified DNA was next sequenced. Basic Local Alignment Search Tool (BLAST) was used to check for sequence matches.

Exhaustion Sporulation

Bacillus anthracis isolated from the appendix of a rabbit (Severson, 2010) was grown in 5% sheep blood tryptic soy agar overnight at 37°C. One to three colonies were picked and grown for 48 hours in 10mL Difco Sporulation Medium (DSM) with supplements (1.2% MgSO₄; 10% KCl; 1N NaOH; 1M Ca (NO₃)₂; 0.01M MnCl₂; 1mM FeSO₄; pH~7). The culture was next diluted 1:5 in DSM medium and allowed to grow for 4 to 5 hours (stationary phase of culture). The liquid culture was diluted a second time at a 1:5 ratio (log phase of culture) and allowed to grow for 24 hours. The culture was centrifuged at 10,000g for 10min at 4°C (RC5C Sorvall Instruments Centrifuge; SS-34 rotor), and the pellet was re-suspended in cold autoclaved distilled H₂O. This was repeated 4 more times. The purified preparation of spores was stored in water at 4°C.

Immuno-dot Blot Analyses

A dot blot apparatus was assembled, packed with filter paper and a nitrocellulose membrane (0.2 μ m Trans Blot Transfer Medium), and connected to a vacuum. Freshly purified wildtype or *Abcla B. anthracis* spores were either sonicated in 1X PBS, or lysed in loading buffer (SDS; 10% 2-Mercaptoethanol). The lysates were further diluted 1:6 in

TBST (Tris buffered saline; Tween 20) buffer, and centrifuged at maximum velocity for 10 minutes in the cold to discard unwanted molecules. The nitrocellulose membrane was loaded with 50 μ L of sonicated or lysed spores, and the vacuum was applied to pull down the proteins into the membrane. The blot was blocked with 5% non-fat milk in TBST overnight. The next day the membrane was probed with 10 μ g/mL a⁺Fv-Ig or Fc γ , followed by 0.8 μ g/mL HRP conjugated donkey anti-rabbit IgG (H+L) (1:2000). The immune-blots were developed with Pierce[®] ECL Western Blotting Substrate (Thermo scientific Lot# NI172907).

Native gel electrophoresis

A 6% native gel was prepared in the absence of SDS. A 10 ml separating gel mixture was polymerized for 1 hour by mixing 40% acrylamide:bis solution (37.5:1), separating gel buffer (36.3g Tris, 150mL dH₂O, pH 8.8), 50% glycerol, dH₂O, 10% ammonium persulfate and TEMED (N, N, N', N'-tetramethyl-ethylenediamine). The stacking gel solution was prepared by mixing 40% acrylamide:bis solution (37.5:1), stacking gel buffer (15.1g Tris, 40mL dH₂O, pH 6.8), 10% ammonium persulfate and TEMED.

Wildtype or mutant *Abcla B. anthracis* spores were dissolved in 2X sample buffer (stacking gel buffer, 20% glycerol, 0.02% Bromophenol Blue, 5% Bromophenol Blue and deionized H₂O) and loaded into the gel. The samples were run at 150V and later transferred overnight into a nitrocellulose membrane. The blot was probed with 40 μ g/mL a⁺FvIg, followed by 0.8 μ g/mL HRP conjugated donkey anti-rabbit IgG (H+L)

(1:2000). The immune-blot was developed with Pierce[®] ECL Western Blotting Substrate (Thermo scientific Lot# NI172907).

Immunofluorescence microscopy

Newly obtained *Δbcla B. anthracis* spores - no older than one week - were washed 3 times with cold autoclaved water, and diluted at a ratio of 1:6. Superfrost[®] Plus slides (VWR Lot# 27999) were coated with poly-L-lysine solution (Sigma-Aldrich Lot# 127K4348), washed 2 times with water, and left to dry overnight. A small aliquot (20μL) of the spores was added to a circular area previously outlined on the surface of the slides using a Super PAP Pen (Immunotech, Beckman Coulter). After 5 minutes of incubation the slides were washed by submersion 3 times in 1X PBS, and air-dried for 30 minutes. The samples were re-hydrated for 5 minutes and washed 2 more times in 1X PBS. The sample were next blocked with 2.5% BSA (Hyclone[®] Thermo Scientific Lot# 110913941E) diluted in 1X PBS for 30 minutes. The samples were stained first with 20μg/mL a⁺FvIg or a⁻FvIg for 1 hour, and then stained with 12.5μg/mL mouse anti-rabbit IgG (BD Pharmingen[™] Lot# 56990) for 30 minutes, followed by 10μg/mL Alexa Fluor[®] 568 goat anti-mouse IgG₁ (γ1) (Invitrogen Lot# 53900A) for 30 minutes. The spores were washed 3 times after the antibody incubations. The sample area was covered with a coverslip after receiving one drop of PermaFluor aqueous mounting media (Thermo Scientific Lot# FM100602). The spores were visualized using a phase-contrast objective on a Leica DM IRB fluorescence microscope equipped with MagnaFire CCD camera.

Biotinylation of proteins

An analytical scale was used to weight enough Biotin (EZ-Link Sulfo-NHS-LC-Biotin Product, No. 21335 Thermo Scientific) to have a final concentration of 2mg/mL Biotin in 1X PBS. The Biotin was added to a tube containing 100µg/mL of a⁺Fv or a⁻Fv. After mixing well, the proteins were left at room temperature for 1 hour. The proteins were next dialyzed in a 0.1-0.5mL dialysis cassette (Slide-A-Lyzer 10K Product No. 66383; Thermo scientific) in 1X PBS overnight to eliminate excess Biotin. The 1X PBS buffer was changed 4 times. The biotinylated proteins were collected the next day and their O.D. was measured.

Immunoprecipitation

Bacillus pumilus, *Propionibacter acnes* or *Bacillus thuringiensis* grown in LB or 5% sheep blood agar plates in modified anaerobic chambers (OXOID, Model G, code HP0031A. GasPakTM EZ Gas Generating Container Systems, BD Lot # 2031360) were washed and re-suspended in 1X PBS. Bacteria were next labeled with 2mg/mL EZ-Link Sulfo-NHS-LC-Biotin for 1 hour at 4°C. After washing with 0.2M Tris in 1X PBS, the labeled bacteria were re-suspended in lysis buffer (1.5M NaCl; 1% NP-40; 1% Triton X-100) and incubated at 4°C for 1 hour. The lysates were centrifuged at 12,500rpm for 2 minutes, the supernatant transferred to clean tubes, and the pellets washed once with 1X PBS to reduce the final salt. The supernatants were pre-cleared two times for 30 minutes, each at 4°C using Protein G beads (GammaBindTM G SepharoseTM protein; GE

Healthcare; Lot 10055876). The supernatants were pooled in clean tubes and stored at -20°C.

At the same time, 30µg/mL a⁺FvIg or a⁻FvIg molecules were conjugated for 40 minutes at 4°C to Protein G beads. In a different experiment, serum from rabbit, human or goat (300µL) was incubated with 100µL of Protein G beads for 30 minutes at 4°C to obtain IgG-Protein G conjugates. The biotinylated bacterial molecules were immunoprecipitated with a⁺FvIg and a⁻FvIg molecules, or with rabbit, human or goat serum IgG for 40 minutes at 4°C.

Western Blot

For immunoprecipitation experiments, the pellet of Protein G-beads conjugated to either FvIg or IgG molecules was washed two times with TBST, and re-suspended in TBST and loading buffer (4X SDS or 2X SDS; 10% 2-Mercaptoethanol). The samples were electrophoresed on 10% SDS-polyacrylamide gels. The precipitated proteins were transferred to Immobilon-P Transfer Membrane (Millipore, Bedford, MD) and probed with 0.1µg/mL HRP conjugated to Streptavidin. The immune-blots were developed with SuperSignal[®] West Pico Chemiluminescent Substrate (Thermo scientific Lot# ND172889).

For *B. anthracis* *Abcla* experiments, the spores were lysed under reducing and non-reducing (with 2-ME or without 2-ME) conditions, and loaded on 12% SDS-polyacrylamide gels. The blots were probed with 15µg/mL a⁺FvIg or a⁻FvIg, followed by 0.8µg/mL HRP-conjugated donkey anti-rabbit IgG (H+L) (1:2000). The immune-blots

were developed with Pierce® ECL Western blotting substrate (Thermo scientific Lot# NI172907).

V_H4 cloning

The constructs for a⁺FvIg (*V_H1a2*, V_{Ha} allotype) and a⁻FvIg (*V_{Hy}33*, V_{Hn} allotype) were originally ligated into pcDNA3.1(-) plasmids of size 5.4 kb (Severson, 2010). The FvIg inserts contain the whole *VDJ* region of the heavy chain of a normal rabbit, a (Gly₄/Ser₁)₃ linker, a *V_κ* gene, and part of the Fc region (Cγ2 and Cγ3) (Fig. 1). The plasmid containing the V_{Ha} construct was used as template to substitute its *V_H1* for *V_H4*, a rearranged gene prevalently found in V_{Ha} cells of *Alicia* rabbits. These two *V_H* genes differ only in 9 base pairs (bp).

To start the cloning process, a 115 bp product extending from framework region 3 (FR3) to the first half of the linker was amplified by polymerase chain reaction (PCR) using primers P1 and P2 (Table 1). Simultaneously, the *V_H4* gene from an *Alicia* rabbit that included the V_H leader was amplified by PCR using primers P3 and P4 (~330bp). The plasmid containing the V_H4 construct was provided by Greg Robins, PhD. These two PCR products were ligated after digesting their respective 3' and 5' regions with the restriction enzyme SacII. The ligated product (~445bp) was PCR amplified using primers P3 and P2, and extended from the V_H leader of the *V_H4* gene to the first half of the linker.

The template plasmid pcDNA3.1(-) containing the V_{Ha} construct was digested from the V_H leader to the first half of the linker using primers P3 and P2 (~445bp). This

segment was replaced by the insert containing the V_H4 gene (~445bp) by ligation.

Plasmids containing the complete constructs containing the V_H4 gene were transfected into Chinese hamster ovary (CHO) cells.

B cell transfection

A total of 250×10^4 cells/mL PBL-1, 55D1 or 79E cells were washed with 10mL RPMI 1640 medium with no fetal bovine serum (FBS), and re-suspended in 1mL HEPES buffer. The cells were mixed with 10 μ g pNF- κ B- Luc reporting plasmid (5kb, Agilent Technologies, provided by Dr. Chris Wiethoff, Loyola University) and 4 μ g pCEP4 (10kb, Invitrogen), which contains a hygromycin mammalian resistance gene. After electroporation (Cell Porator®, Gibco BRL), the transfected cells were re-suspended in RPMI and added to a 24-well plate. Next, 0.2 mg/mL Hygromycin B selection drug (CALBIOCHEM®, Lot# B50706) was added to the wells and incubated for 2 days at 37°C. The cells were treated with the selection drug and monitored every 2 days in order to find stable transfectants.

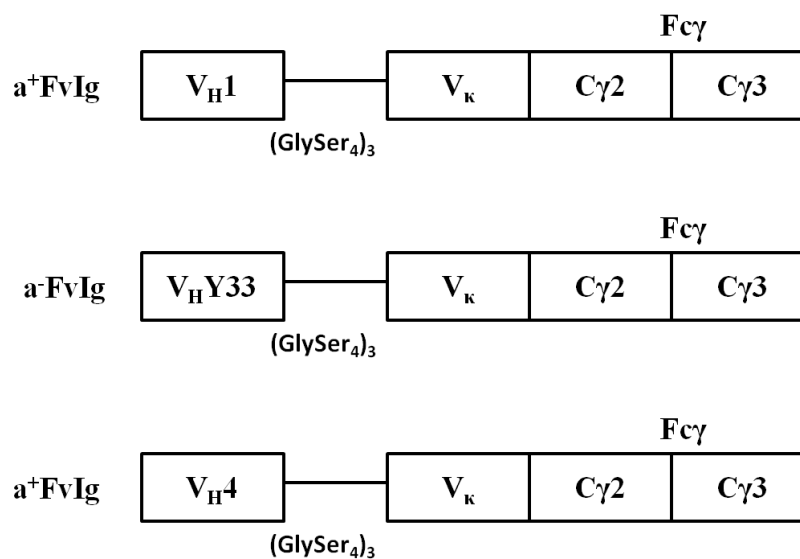


Figure 1: Schematic of FvIg molecules containing different V_H genes.

The top and center part of the figure are the FvIg constructs (a⁺FvIg and a⁻FvIg) designed and developed by K. Severson (2010). The bottom part of the figure shows the Fv molecule containing a V_H4 gene. The constructs contain the *VDJ* region of the heavy chain of a normal rabbit, a (Gly₄/Ser₁)₃ linker, a V_κ gene, and part of the Fc region (Cγ2 and Cγ3).

Table 1: List of primers and their sequences

Primer	Name	Sequence
P1	OS-VH-SacIII	CTG <u>CCGCGG</u> ACACGGCCACC TATTT
P2	OA-VH-LINKER-BAM2	TGGATCCACCGCCACCTGAG GAGACGGTGACCAG
P3	OS-V-LDR-XHO	ACTCGAGGCGCCACCATGG AGATT
P4	OA-VH4-FR-SACII	TGT <u>CCGCGG</u> CTGTCAGACTG GTCA
P5	16sRNApan27f	AGAGTTTGATCCTGGCTCAG
P6	16sRNApan1492r	CGGTTACCTTGTTACGACTT

Luciferase detection

Approximately 1×10^6 55D1 cells, transfected with an NF- κ B Luciferase plasmid, were stimulated with goat anti-rabbit IgM at 1:5, 1:10 and 1:20 dilutions, or with sonicated *B. pumilus* for 20 minutes, 2 hours and 4 hours. The cells were washed at 1000rpm for 5 minutes at room temperature with 1X PBS. The pellet was re-suspended in 100 μ L 1X Passive Lysis Buffer (PLB) and placed in a rocker for 15 minutes. A volume of 25 μ L of cell lysate was loaded into a white 96-well plate. The lysates were read in a Veritas Microplate Luminometer (Turner Biosystems), using a Luciferase Assay Substrate (Promega Lot # 0000027225).

Stimulation of B cells

Rabbit stable B cell lines - PBL-1, 55D1 and 79E - were stimulated with different molecules in the presence or absence of CD40L expressed on the surface CHO cells. The molecules used for stimulation are as follow: goat anti-rabbit IgM (serum, Maggie) at a 1:5, 1:10 or 1:20 dilution; sonicated *B. pumilus* (200 μ L); 5 μ g/mL of purified exopolysaccharide from *B. subtilis* (sinRtasA or sinRtasAepsH); 10 μ g/mL of TLR2,6 agonist Pam3CSK4 Lyophilized (InvivoGen Lot# 28-04-PMS); 10 μ g/mL of TLR1,2 agonist Pam2CSK4 (InvivoGen Lot# PM2-33-03); 1 μ g/mL LPS from *Salmonella enterica* serotype *enteritidis* (Sigma Aldrich Lot# L7770-1MG).

CHAPTER THREE

RESULTS

ROLE OF BACTERIAL MOLECULES IN B CELL ACTIVATION AND MIGRATION IN GALT

In rabbits, antibody diversity is generated in GALT where B cells migrate after leaving the BM. B cells that seed GALT expand polyclonally and the primary antibody repertoire expands. These processes require interaction of GALT with intestinal microbiota. However, the molecular mechanism by which bacteria promote GALT development and function is not yet fully understood.

I hypothesized that molecules of selected bacterial species in the intestine of rabbits cause the activation of B cells and also alter the expression of chemokine receptors, which regulates their migration in GALT. To understand the mechanism by which commensals drive GALT reactions, I investigated the processes that lead to the activation and migration of B cells in GALT. Using FvIg recombinant molecules, I asked if bacterial molecules from the intestine of rabbits bind to surface Ig of B cells or to soluble Ig. To determine whether bacteria provide signals for activation and migration, I detected the expression of B7, a surface molecule of the Ig family expressed on activated B cells but not on resting B cells. In addition, I detected the expression of

chemokine receptors (CCR6, CXCR5, CCR7 and CXCR4), known to be expressed on B cells as they move throughout the follicle.

The results of these experiments, as described below, lead me to propose that bacterial products provide B cells with signals that may lead to their activation and relocation to other regions of the follicle.

Binding of intestinal bacteria to FvIg

The intestinal microbiota has a role in the generation and diversification of B cells in GALT. Serum Ig secreted from B cells of normal rabbits shows a predominant V_{HA} allotype over the V_{HN} allotype. In contrast to this, *Alicia* mutant rabbits show at birth a prevalence of the V_{HN} allotype. Upon contact with the gut microbiota, the mutant rabbit V_{HN} B cell population stops proliferating and instead, V_{HA} B cells expand. These events may take place by selective expansion induced by bacteria. This is supported by the fact that rabbits with surgically ligated appendix (germ-free) fail to develop V_{HA} B cells (Rhee, 2004).

The initial goal of this research was to determine if the repertoire expansion from V_{HN} to V_{HA} B cells results from interactions between a putative bacterial superantigen with V_{HA} B cells in the GALT of *Alicia* rabbits. To test this original hypothesis I used biotinylated a⁺FvIg (**V_{HA}**) and a⁻FvIg (**V_{HN}**) recombinant proteins to isolate bacterial species from the intestine of rabbits. Bacteria binding to the FvIg were detected with APC conjugated Streptavidin. Figure 2 shows three groups of bacteria that bind to either

a⁺FvIg (blue events), a⁻FvIg (green events), or to both FvIg simultaneously (purple events). Biotinylated Fcγ was used as negative control. The P1 population displayed in the left side panel did not bind to Fcγ and was used for the subsequent sorting. Only bacteria binding to a⁺FvIg and a⁻FvIg were used for later experiments. Bacteria binding to both FvIg at the same time (double population; purple events) were dismissed.

I initially obtained 12 bacterial isolates from the intestines of rabbits that grew in media, and were later identified by 16sRNA sequencing. Table 2 displays the names of each species, their gram stain, and their ability to produce spores. This table also shows whether the species were initially sorted with a⁺FvIg or a⁻FvIg, and if they grew in aerobic or anaerobic conditions. To test if the binding profile of these bacteria to the FvIg molecules was reproducible, I re-screened the isolates by FACS using the FvIgs a minimum of three times. The results of the re-screening experiments showed that most of the bacterial species no longer bound to the FvIgs (data not shown). Only 3 species - *B. thuringiensis*, *B. pumilus* and *P. acnes* - retained their binding activity to the FvIg molecules. These 3 organisms were further tested.

Figure 3 shows two independent re-screenings of *B. thuringiensis* using FvIgs. This species was initially sorted with a⁺FvIg. On the first experiment (Fig 3A) I was able to reproduce the initial sorting profile: *B. thuringiensis* bound to a⁺FvIg (green histogram) but not to a⁻FvIg (purple histogram). However, in a later experiment, this specie did not bind to either one of the FvIgs (Fig. 3B).

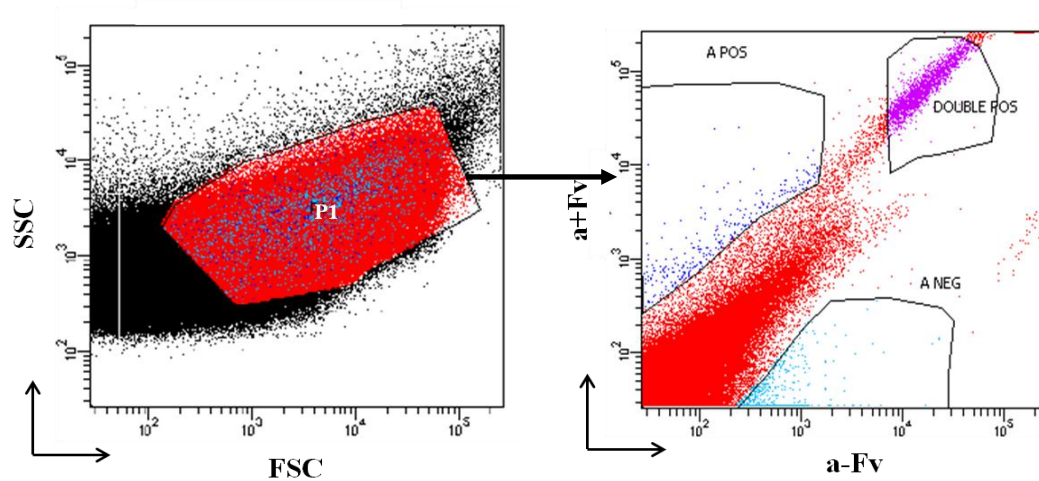


Figure 2: Flow cytometric analysis of bacteria from the intestine of rabbits

bound to FvIg. The contents of the intestine of rabbits were centrifuged to remove debris and fecal material. The supernatant was stained with 10 μ g/mL Fc γ biotinylated a⁺FvIg and a⁻FvIg. The gated P1 population (left panel) did not bind to the negative control Fc γ . The right hand panel shows cells binding to a⁺FvIg (blue dots); cells binding to a⁻FvIg (green dots); and cells binding both Fv (purple dots). The cells were detected with APC conjugated Streptavidin, and sorted by FACS Aria cell sorter.

Table 2: Characteristics of isolated bacterial species and their sorting profile

Specie	Gram stain	Spore former	Sorted	Culture condition
<i>Neisseria subflava</i>	negative	no	a ⁺ FvIg	aerobic
<i>Caldimonas manganoxidans</i>	negative	no	a ⁺ FvIg	aerobic
<i>Propionibacter acnes</i>	positive	no	a ⁺ FvIg	aerobic
<i>Bacillus thuringiensis</i>	positive	yes	a ⁺ FvIg	aerobic
<i>Neisseria subflava u37</i>	negative	no	a ⁻ FvIg	aerobic
<i>Neisseria flavescens</i>	negative	no	a ⁻ FvIg	aerobic
<i>Propionibacterium acnes</i>	positive	no	a ⁺ FvIg	anaerobic
<i>Bacillus aerophilus 28K</i>	positive	yes	a ⁺ FvIg	anaerobic
<i>Bacillus pumilus attc7061</i>	positive	yes	a ⁺ FvIg	anaerobic
<i>Bacillus safensis</i>	positive	yes	a ⁻ FvIg	anaerobic
<i>Propionibacterium acnes</i>	positive	no	a ⁻ FvIg	anaerobic
<i>Neisseria flavescens N155</i>	negative	no	a ⁻ FvIg	anaerobic

Since I was able to grow *B. thuringiensis* in both aerobic and anaerobic conditions, I tested if this factor would influence binding to FvIgs. The results obtained in Figure 4 suggested that this was correct. When cultured in liquid broth under aerobic conditions, *B. thuringiensis* lost its ability to bind to the FvIg molecules (Fig. 4C). However, when grown in liquid broth under anaerobic conditions most of the cells bound to a⁺FvIg but not a⁻FvIg (Fig. 4 D). Strikingly, I observed that this organism can also bind to a⁻FvIg (Fig. 4, A&B), contradicting the initial sorting and the re-screening results shown in Figure 3.

I performed similar re-screens on *B. pumilus*, which was initially sorted after binding to a⁺FvIg, and grew exclusively under anaerobic conditions. To test if this organism would replicate its initial binding to a⁺FvIg, I performed independent experiments using the same conditions. I initially confirmed that *B. pumilus* binds a⁺FvIg (green histogram) but not to a⁻FvIg (purple histogram) (Fig. 5A). However, a latter experiment (Fig. 5B) demonstrated that this species can also bind to a⁻FvIg (green histogram) but not to a⁺FvIg (blue histogram) or to Fc γ (brown histogram). Since I had previously observed that the condition of the culture may influence the binding of *B. thuringiensis* to FvIgs (Fig. 4), I tested if the same was the case for *B. pumilus*. I cultured the cells on blood agar plates or in a broth under anaerobic conditions and stained them with FvIgs. I found that under both growth conditions, over 66% *B. pumilus* cells bound a⁺FvIg (Fig. 6, A&B).

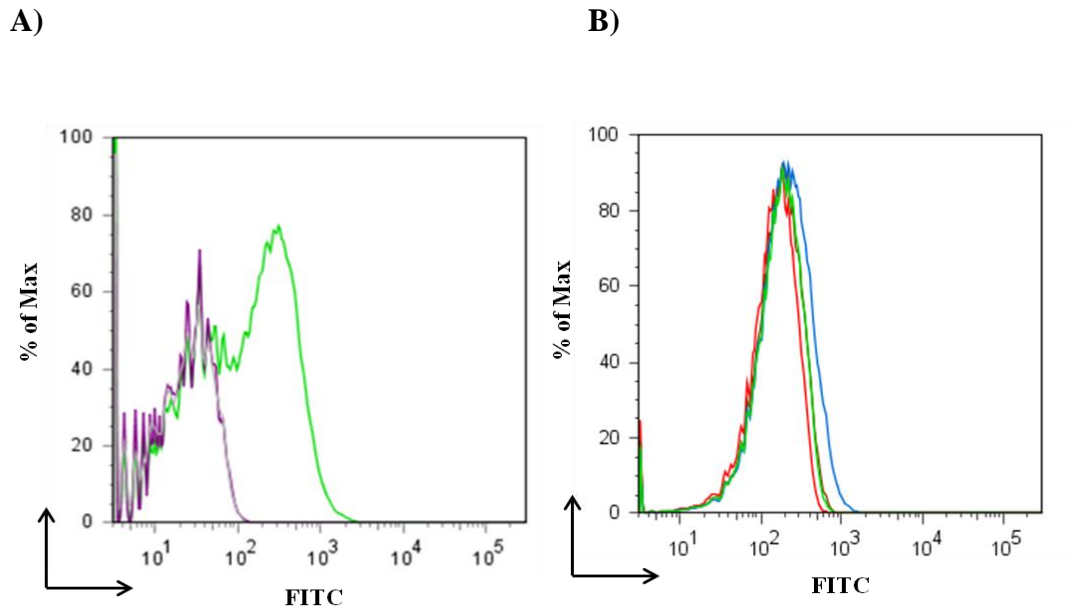


Figure 3: Flow cytometric analysis of *Bacillus thuringiensis* binding to FvIg.

Two different experiments, two months apart: A) former experiment using cells from an anaerobic blood agar plate. Bacteria were stained with 20μg/mL FITC-conjugated a⁺FvIg (green), FITC-conjugated a⁻FvIg (purple), or left unstained (gray). B) Later experiment using cells from an aerobic blood agar plate.

Bacteria were stained with 20μg/mL FITC-conjugated a⁺FvIg (green), FITC-conjugated a⁻FvIg (blue), FITC-conjugated Fcγ (brown), or left unstained (red).

The analysis was performed in a LSR Fortessa[™] flow cytometer.

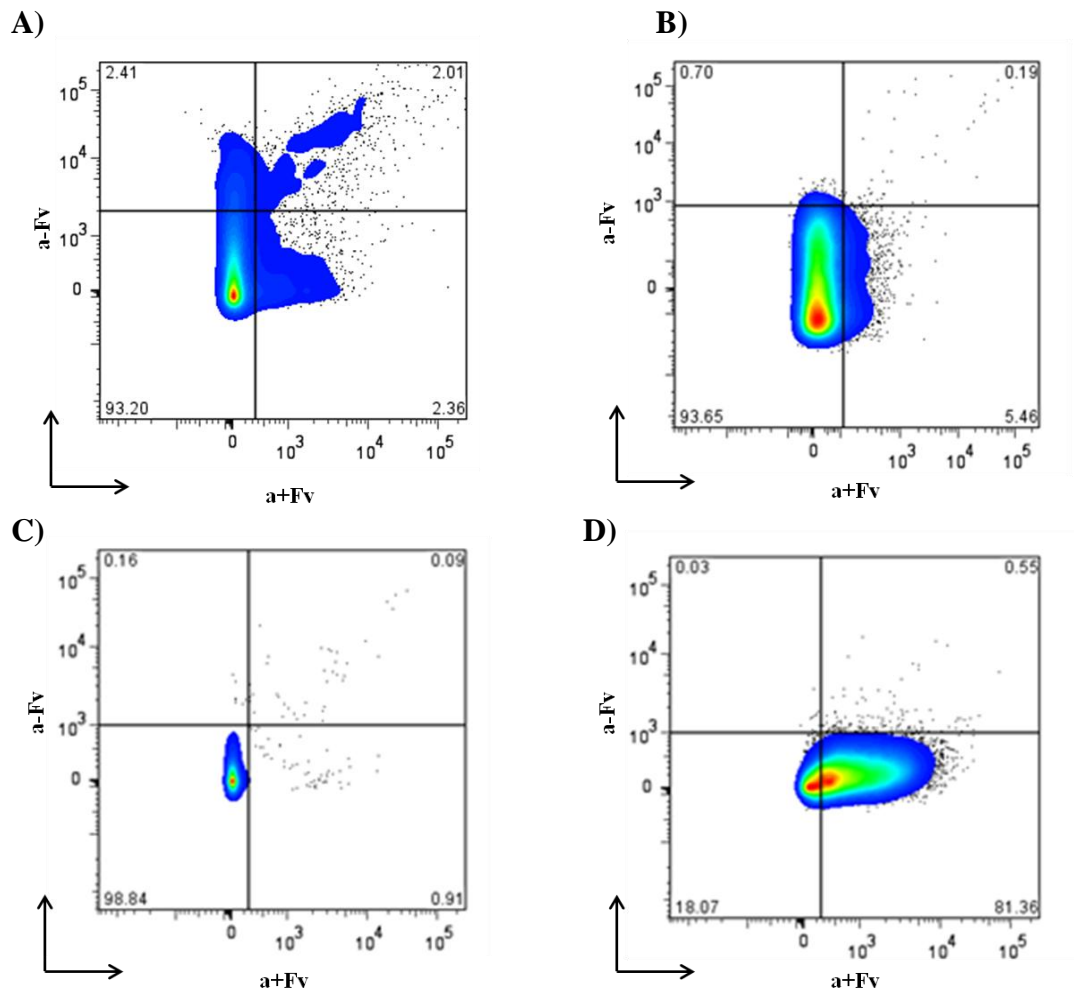


Figure 4: Flow cytometric analysis of *Bacillus thuringiensis* binding to FvIg.

Bacteria cells were cultured under different conditions: A) anaerobic blood agar plate, B) aerobic blood agar, C) aerobic broth and D) anaerobic broth. Bacteria were stained with 20µg/mL APC-conjugated a⁺FvIg or PE-conjugated Cy7-a⁻FvIg. Each experiment was performed independently over the course of 2 weeks. The analysis was performed in a LSR FortessaTM flow cytometer.

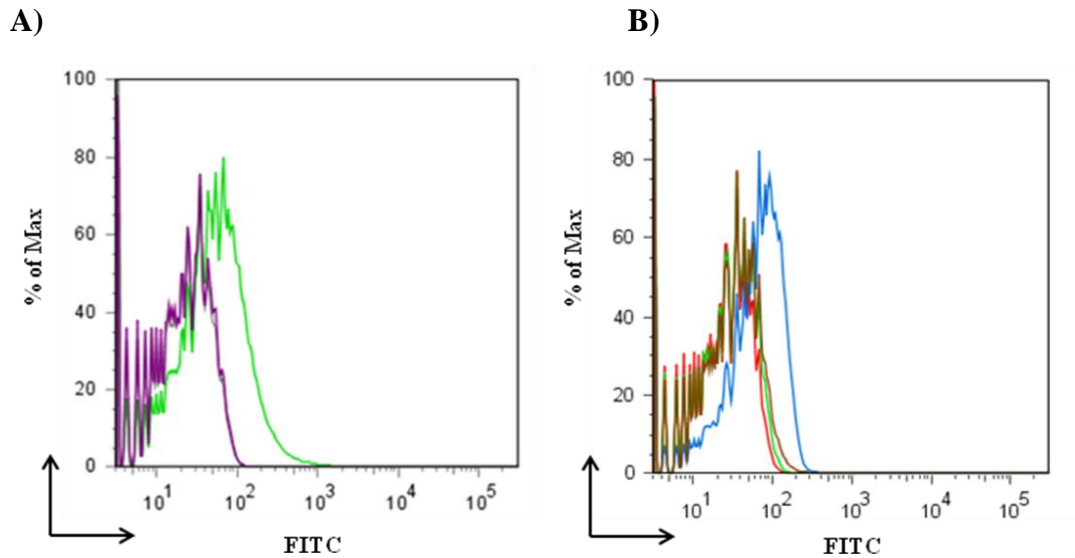


Figure 5: Flow cytometric analysis of *Bacillus pumilus* binding to FvIg. Two different experiments, two months apart: A) First experiment using cells from an anaerobic blood agar plate. Bacteria were stained with 20μg/mL FITC-conjugated a⁺FvIg (green line), FITC-conjugated a FvIg (purple line), or left unstained (gray line). B) Second experiment using cells from an anaerobic blood agar plate. Bacteria were stained with 20μg/mL FITC-conjugated a⁺FvIg (green line), FITC-conjugated a FvIg (blue line), FITC-conjugated Fcγ (brown line), or left unstained (red line). The analysis was performed in a LSR Fortessa™ flow cytometer.

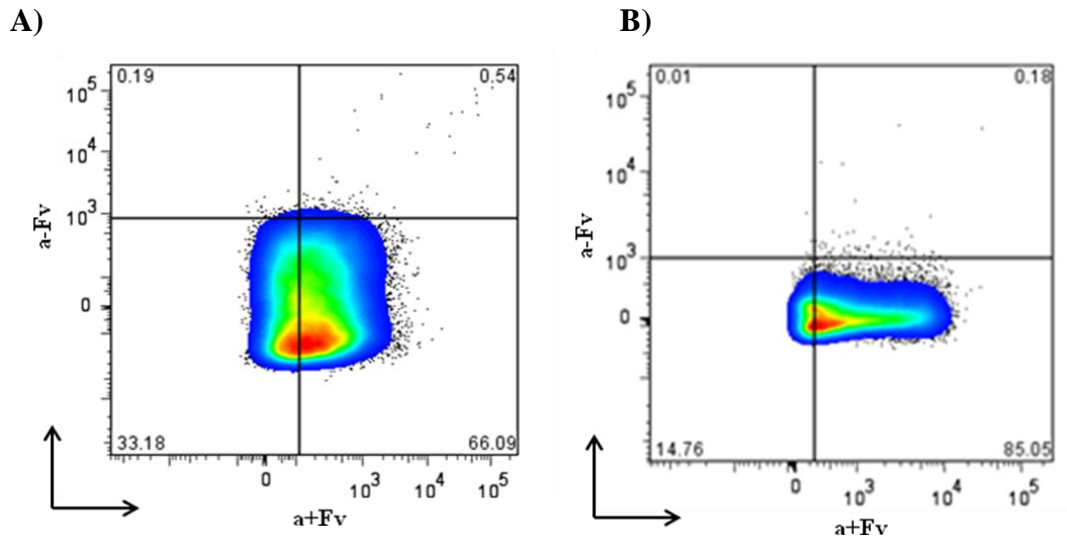


Figure 6: Flow cytometric analysis of *Bacillus pumilus* binding to FvIg.

Bacterial cells were cultured under different conditions: A) anaerobic blood agar plate or B) anaerobic broth. Bacteria were stained with 20 μ g/mL APC-conjugated a⁺FvIg or PE-conjugated Cy7-a⁻FvIg. Each experiment was performed independently over the course of 2 weeks. The analysis was performed in a LSR FortessaTM flow cytometer.

The third organism of interest *P. acnes* was initially sorted with a⁻FvIg and grew exclusively under anaerobic conditions. Further experiments showed that this organism, re-screened with a⁺FvIg (Fig. 7, A&B), did not bind to a⁻FvIg. In both experiments, over 57% of the cultured cells bound to a⁺FvIg. However, at least one independent experiment showed that *P. acnes* did not bind to either FvIg molecule (Fig 8).

The data collected in these experiments showed that bacterial molecules can bind Ig, but that the binding of bacteria from the intestines of rabbits to FvIg varies with culture conditions. In addition, I concluded that none of the isolated bacterial species bind exclusively to either a⁺FvIg or a⁻FvIg.

Detection of bacterial molecules binding to FvIg

A previous study in the Knight Lab suggested an interaction between *Bacillus* spores and Igs. In this study, K. Severson (2010) observed that both FvIg and rabbit IgM bound robustly to *B. anthracis* $\Delta bclA$ mutant spores. This mutant strain lacks spore surface hair-like projections, and has therefore, an exposed exosporium. The study goes on to suggest that the FvIgs bind to epitopes on the exosporium. To confirm this hypothesis, they tested a *B. anthracis* mutant strain lacking the exosporium ($\Delta cotO$) and found, by Western blot, that FvIg would bind to a molecule of ~25kDa from wildtype (WT spores); however, FvIg did not bind to the mutant lacking the exosporium. The study identified the ~25kDa molecule as ExsK, which is known to be present in the

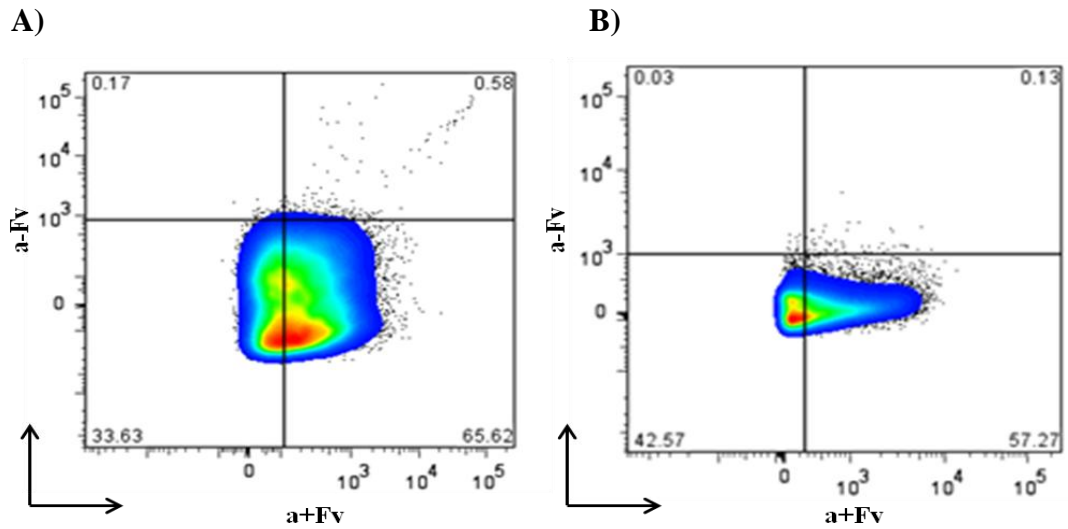


Figure 7: Flow cytometric analysis of *Propionibacter acnes* binding to FvIg.

Bacteria cells were cultured under different conditions: A) aerobic blood agar plate, B) anaerobic blood agar plate. Bacteria were stained with 20 μ g/mL APC-conjugated a⁺FvIg or PE-Cy7-conjugated a⁻FvIg. Each experiment was performed independently over the course of 2 weeks. The analysis was performed in a LSR FortessaTM flow cytometer.

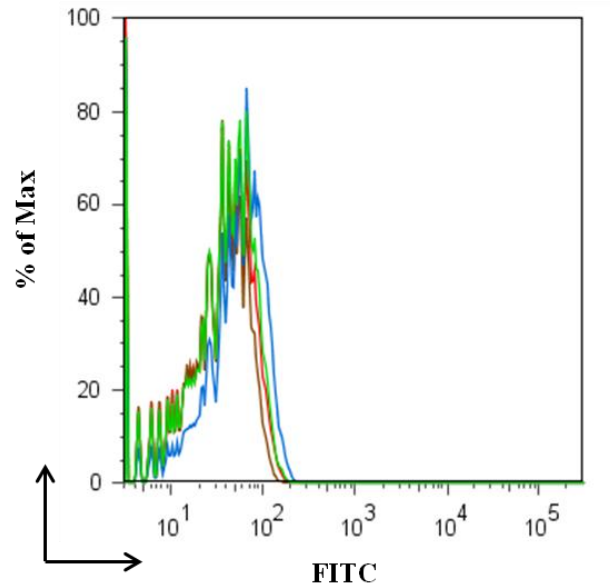


Figure 8: Flow cytometric analysis of *Propionibacter acnes* binding to FvIg.

Bacteria from an anaerobic blood agar plate were stained with 20μg/mL FITC-conjugated a⁺FvIg (green line), FITC-conjugated a⁻FvIg (blue line), FITC-conjugated Fcγ (brown line), or left unstained (red line). The analysis was performed in a LSR Fortessa[™] flow cytometer.

exosporium of spores. It was also concluded that both FvIg and rabbit IgM bind to ExsK on the surface of *B. anthracis* (Severson, 2010). Therefore, I reasoned that *B. anthracis* $\Delta bcla$ was an appropriate positive control for my binding experiments using FvIgs.

Testing $\Delta bcla$ control by Western blot, Dot blot and Native gel electrophoresis

I showed by flow cytometry that bacteria from the intestines of rabbits bind to FvIgs. However, I did not know if one or more molecules are binding to these recombinant molecules. Moreover, I did not know the identity of such molecule(s). To detect the bacterial molecule(s) binding to the FvIgs, I made bacterial lysates and performed Western blot assays using FvIgs.

The first logical step was to determine if the positive control *B. anthracis* $\Delta bcla$ would work in my system. This mutant was shown to bind to the FvIgs by flow cytometry experiments and by immuno-fluorescence microscopy experiments, but it had not yet been tested by western blot. I made lysates of purified WT and mutant *B. anthracis* spores using SDS with 2-ME and performed a western blot. I probed the bacterial samples with either a⁺FvIg or a⁻FvIg. If *B. anthracis* ExsK molecule binds to FvIgs, then by using the $\Delta bcla$ mutant, which has an exposed exosporium, I would expect to find bands of about 12kDa and 25kDa, the sizes of the ExsK molecule. The goal of this experiment was to reproduce the results previously obtained in the Knight Lab, this time using western blot analysis.

Figure 9A shows a western blot of proteins from WT and *Abcla* spores prepared under reducing and non-reducing conditions. The blot was probed with a⁺FvIg. I observed faint high molecular weight proteins (lines 3 and 4) from the *Abcla* mutant spores, but was unable to observe 12kDa or 25kDa bands. I also did not detect proteins in the WT spore lysate samples (lines 1 and 2). To determine if the ExsK protein was instead binding to a FvIg, I probed a blot with this recombinant molecule (Fig. 9B) and found similar results to those obtained when probing with a⁺FvIg. I observed a few high molecular weight proteins (lines 3 and 4) in the *Abcla* mutant spore samples, but I was unable to observe 12kDa or 25kDa bands. Further, I also did not detect proteins in the WT spore lysate samples (lines 1 and 2).

The results shown in Figure 9 are a representation of several unsuccessful experiments designed to detect ExsK by western blot analysis. One possibility to explain this is that ExsK was not able to bind FvIg molecules when tested in a denatured/unfolded state. To address this possibility, I designed and performed Dot blot assays. This approach gave me a tool to test simultaneously reduced/denatured and unreduced/native *B. anthracis* spore samples.

The top half of Figure 10 shows WT and *Abcla* spore lysates that were reduced using conventional SDS loading buffer in the presence of 2-ME. The lower half of both panels show WT and *Abcla* spore lysates that were previously sonicated in 1X PBS and kept in an unreduced/non-denatured state. The blots were probed with a⁺FvIg (Fig. 10A)

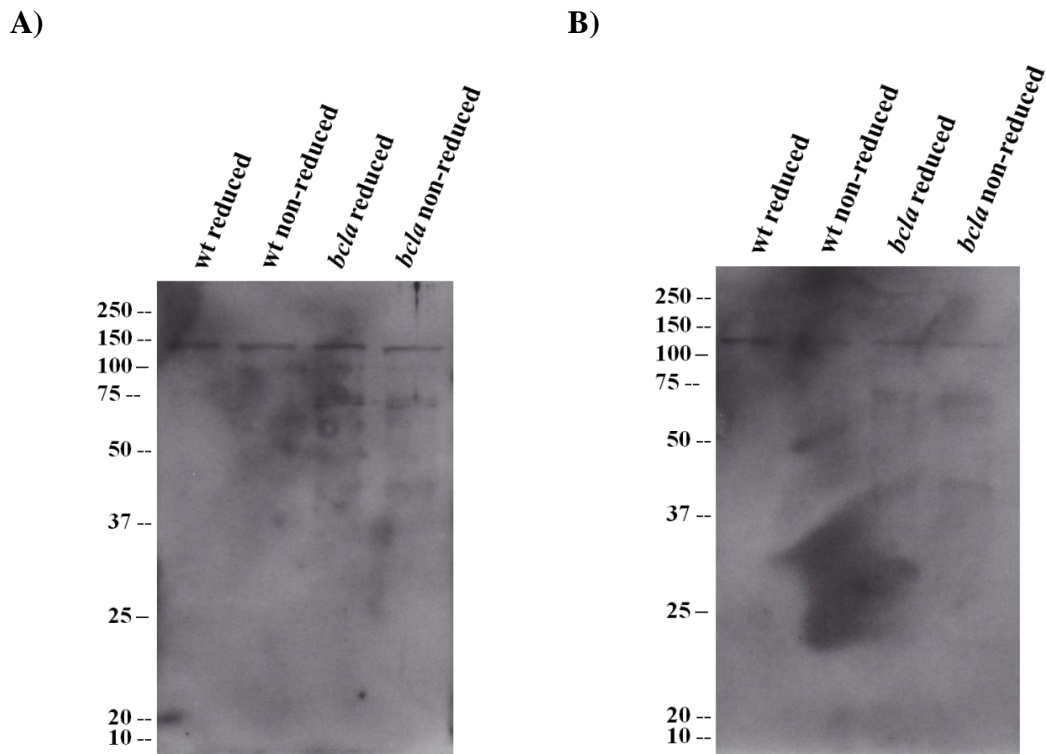


Figure 9: Western blot analysis of *B. anthracis* spores binding to FvIg.

Wildtype or $\Delta bclA$ mutant spores were lysed under reduced or non-reduced (with 2-ME or without 2-ME) conditions, and loaded on a 12% SDS-polyacrylamide gel. The blots were probed with A) 15 μ g/mL a⁺FvIg or B) 15 μ g/mL a⁻FvIg. The FvIg molecules were detected with 0.8 μ g/mL HRP-conjugated Donkey anti-Rabbit IgG (H+L) (1:2000).

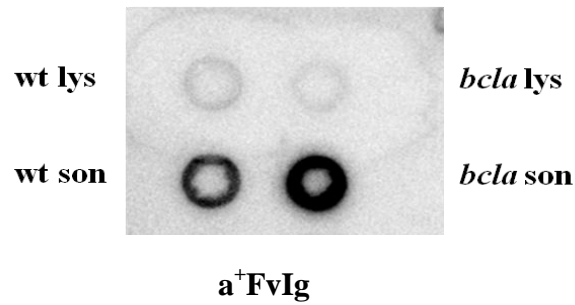
or Fc γ (Fig. 10B), and developed using the conventional western blot method. Proteins were only detected in the sonicated WT and *Abcla* samples by a⁺FvIg (Fig. 10A, black dots) but not by Fc γ .

To further test whether the native state of ExsK determines its binding to FvIg I designed and performed native gel electrophoresis experiments. In these experiments the gel and electrophoresis solutions were prepared without SDS and without the reducing agent dithiothreitol (DTT), used in standard SDS-PAGE. In this assay the protein of interest retains its folded conformation. However, after several attempts to detect ExsK with FvIgs under native conditions, I did not have definitive results that would confirm that the native state of ExsK determines binding to FvIg. One of such attempts is depicted in Figure 11. Wildtype and *B. anthracis Abcla* spores were dissolved in non-denaturing conditions and loaded on a 6% native gel. The blots were probed with a⁺FvIg (left panel), a⁻FvIg (center panel), or Fc γ (right panel) as negative control. I was not able to detect ExsK or any other spore protein in any of the blots.

Immunofluorescence of B. anthracis spores

Previous immunofluorescence experiments performed in the Knight Lab demonstrated that *B. anthracis Abcla* mutant spores, but not to WT spores, bind to FvIg and IgM. These studies also suggested that this binding is mediated by the ExsK protein exposed on the surface of the *Abcla* spores. Additionally, I have gathered evidence that

A)



B)

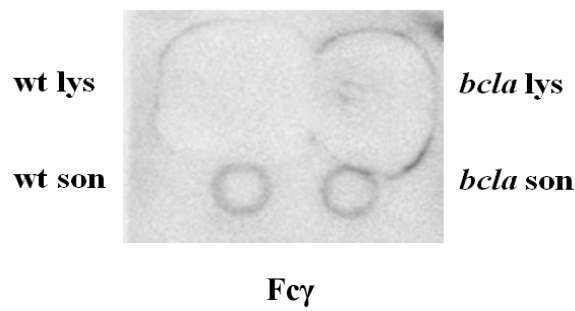


Figure 10: Dot blot analysis of *B. anthracis* spores binding to FvIg. Wildtype or $\Delta bcla$ mutant spores were lysed under reducing conditions (w/ 2-ME) or prepared under non-reducing native conditions (sonication in 1X PBS). The samples were loaded on a 0.2 μ m nitrocellulose membrane and probed with A) 10 μ g/mL a^+ FvIg or B) Fc γ . The proteins were detected with 0.8 μ g/mL HRP conjugated donkey anti-rabbit IgG (H+L) (1:2000).

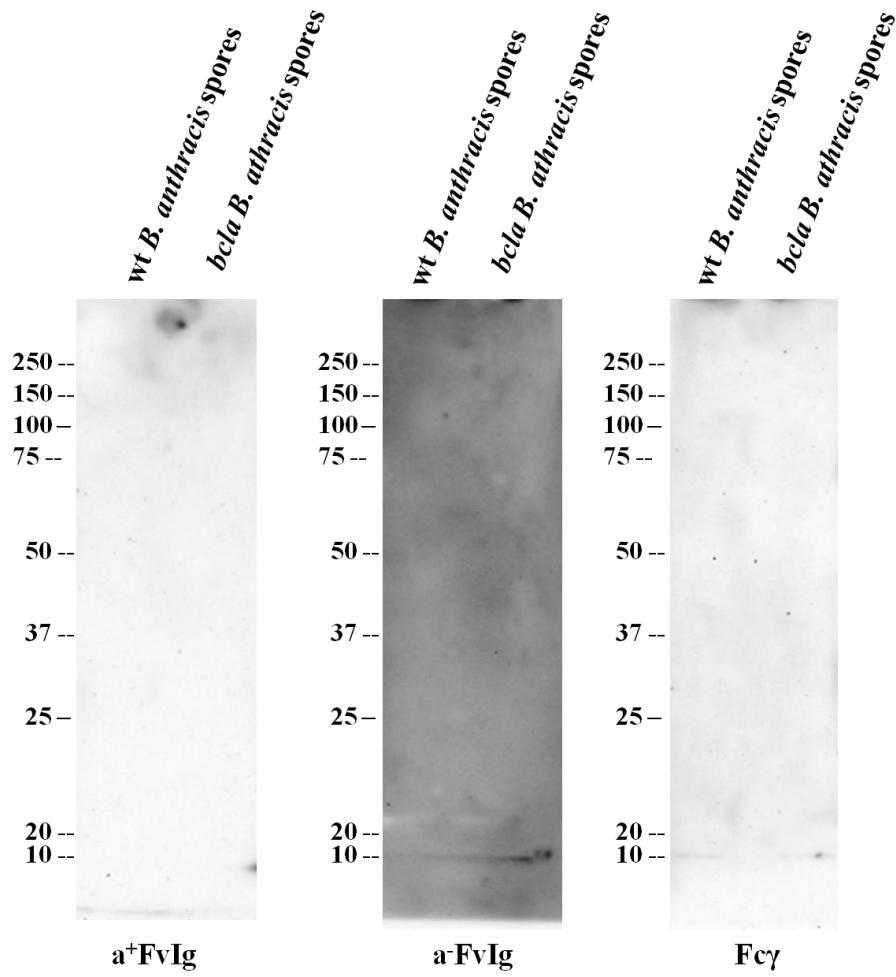


Figure 11: Native gel electrophoresis of *B. anthracis* spores binding to FvIg.

Wildtype or $\Delta bclA$ mutant spores were dissolved under native conditions. The samples were loaded on a 6% PAGE gel, transferred into a nitrocellulose blot, and probed with 40 μ g/mL a^+ FvIg (left), a^+ FvIg (center), or Fc γ (right). The proteins were detected with 0.8 μ g/mL HRP conjugated donkey anti-rabbit IgG (H+L) (1:2000).

suggest that ExsK or other proteins involved in binding to FvIgs do so only when their native structure is intact (Fig. 10). However, I did not confirm this idea by native gel electrophoresis experiments (Fig. 11). One possibility is that due to human mistake, the spores I used for my western blot experiments (Figures 9, 10 & 11) were actually not *B. anthracis* $\Delta bcla$, but rather spores from other species. To verify in some measure that I was using the correct *B. anthracis* spores, I performed immunofluorescence microscopy experiments. I reasoned that if the spores were not *B. anthracis* $\Delta bcla$, then I would not be able to reproduce the results previously obtained in the Knight Lab, in which *B. anthracis* $\Delta bcla$ mutant spores, but not to WT spores, bound to FvIg and IgM.

I coated microscopy slides with *B. anthracis* WT or with what I thought was $\Delta bcla$ spores, and probed them with a⁺FvIg (Figure 12, A&C) or the negative control, Fc γ (Figure 12, B&D). I expected to observe the putative $\Delta bcla$ spores binding to a⁺FvIg but not to Fc γ . In addition, I expected to find that WT spores do not bind to either a⁺FvIg or Fc γ . The top row of Figure 12 shows phase-contrast images of the spores, while the bottom row shows fluorescence images of the *B. anthracis* spores. The images presented in this figure were captured using the same magnification.

The results of this experiment show that the presumed $\Delta bcla$ spores bind to a⁺FvIg but not to Fc γ (Fig. 12, A&B, bottom). I also observed that WT spores did not bind to either a⁺FvIg or to Fc γ (Fig. 12, C&D, bottom). Overall, my results indicate that the putative $\Delta bcla$ spores, but not to WT spores, bind to FvIg. These results do not

confirm the identity of the spores as *Abcla B. anthracis*; however, it shows that these ‘mystery’ spores bind to FvIg in an identical manner to *Abcla B. anthracis* spores.

Immunoprecipitation of bacterial molecules

As described above, the attempts to detect Ig-binding molecules in intestinal bacteria or spores by western blot were ineffective. To further test for such Ig-binding molecules, I performed immunoprecipitation experiments, which would give me the means to isolate particular protein(s) from a bacterial lysate containing numerous different proteins.

My approach was to first, biotinylate the surface of bacterial species isolated from the intestine of rabbits (Table 2). For this experiment I used the bacteria of interest *B. thuringiensis*, *B. pumilus* and *P. acnes*. This was followed by lysing the cells in a high salt, mild detergent buffer (1.5M NaCl; 1% NP-40; 1% Triton X-100); the lysates were incubated with FvIg conjugated to Protein G beads, and the precipitates were loaded onto an SDS-polyacrylamide gel, and a western blot analysis was performed. If bacterial molecules bind to FvIg, then I would expect to precipitate such molecule(s) with FvIg.

The resulting western blot revealed a ~20kDa bacterial molecule from *B. pumilus* and *P. acnes* that immunoprecipitated with both a⁺FvIg and a⁻FvIg (lines 1 to 4, Figure 13). Binding of this molecule to a⁻FvIg was considerably more abundant than binding to a⁺FvIg. Additionally, I observed that several molecules from *B. thuringiensis* precipitated with both a⁺FvIg and a⁻FvIg (lines 5 and 6). These results suggest that surface molecule(s) from intestinal bacteria of rabbits can bind to FvIg.

Immunoprecipitation of bacterial molecules with IgG from serum of diverse species

The expansion of B cells and somatic diversification of Ig genes in GALT is polyclonal, and occurs in an antigen- and T cell-independent mechanism (Pospisil and Mage, 1998). The process of somatic diversification requires interaction of GALT with intestinal microbiota (Rhee, *et al.*, 2004). At least one study suggests that B cells can be polyclonally stimulated *in vitro* by bacteria through the engagement of IgM by B cell superantigens (Severson, 2010).

These data led me to initially hypothesize that a bacterial superantigen binds to IgM on the surface of B cells, independent of their antigen specificity. With the purpose of testing this hypothesis, I was able to isolate bacteria from the intestine of rabbits using FvIgs (Table 2). In addition, I successfully identified a ~20kDa bacterial molecule from intestinal bacteria that immunoprecipitated with FvIgs (Fig. 13). These data indicated that bacterial molecules can bind surface Ig and supported the idea that in the FAE, bacteria provide B cells with signals that may lead to their activation.

To further test if bacterial molecules can bind to the surface Ig of B cell, I performed additional immunoprecipitation assays and tested if biotinylated *B. pumilus* surface proteins can bind serum IgG. Bacterial cell lysates were incubated with serum IgG from rabbit, human or goat conjugated to Protein G beads. The immunoprecipitation of bacterial molecules was detected by western blot. If bacterial molecules bind to B cell surface Ig to provide signals that may stimulate B cell activation, then I expected that such molecules would immunoprecipitate with serum Igs.

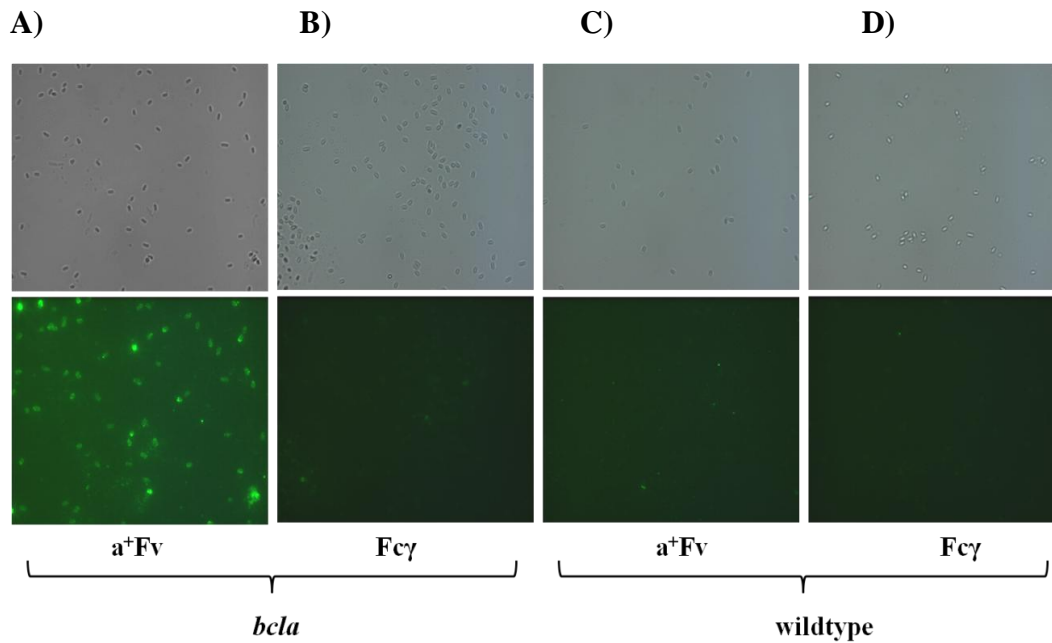


Figure 12: Immuno-fluorescence microscopy of *B. anthracis* spores. *Bacillus anthracis* $\Delta bcla$ spores (A and B) or WT spores (C and D) were fixed onto slides coated with poly-L-lysine solution. The top row shows phase contrast images of the spores. The bottom row shows immuno-stained spores. The samples were stained with 20 μ g/mL a⁺FvIg (A and C) or Fcγ (B and D), followed by 2.5 μ g/mL mouse anti-rabbit IgG. The spores were detected with 10 μ g/mL DyLight488-conjugated goat anti-mouse (Fab), and visualized using a fluorescence microscope equipped with MagnaFire CCD camera. The same magnification was used in each case.

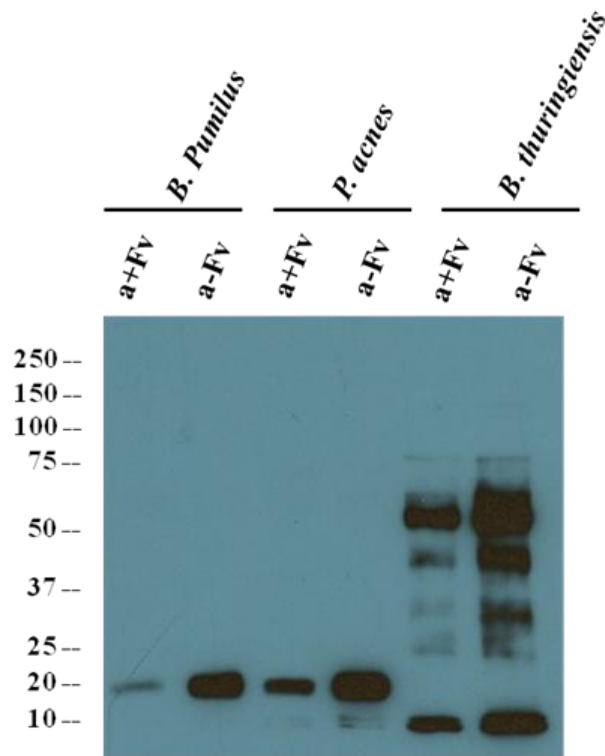


Figure 13: Immunoprecipitation of bacterial molecules with FvIg. *B.*

pumilus, *P. acnes* and *B. thuringiensis* were labeled with 2mg/mL of EZ-Link Sulfo-NHS-LC-Biotin. Bacteria were washed with 0.2M Tris in 1X PBS and lysed in lysis buffer (1.5M NaCl; 1% NP-40; 1% Triton X-100). The biotinylated bacterial molecules were immunoprecipitated with 30μg/mL *a*⁺FvIg and *a*⁻FvIg molecules that had been conjugated to Protein G beads. The precipitates were loaded on an SDS-polyacrylamide gel and western blot was performed. The proteins were detected with HRP-conjugated Streptavidin.

The results of two independent experiments are shown in Figure 14 A and B. In both experiments I observed that not one, but several, bacterial molecules bound to rabbit, human and goat IgG. In addition, I detected slight differences in the size and abundance of bacterial molecules that bind to IgG from these three mammalian species. These results provide further evidence that bacterial molecules can bind Ig and they are consistent with the hypothesis that bacterial molecules can activate rabbit B cells through surface Ig.

Intestinal bacteria bind to Ig

To confirm that bacteria isolated from the rabbit intestine bind to whole Ig, I tested vegetative cells by flow cytometry. I had successfully shown that proteins of *B. pumilus* immunoprecipitate with FvIgs (Fig. 13), and I hypothesized they would also bind to intact Igs. My source of Igs was rabbit serum.

I incubated vegetative *B. pumilus* cells with normal serum from rabbit, and detected the binding profiles of these bacteria to IgA, IgG or IgM (Fig. 15). The results show that *B. pumilus* binds robustly to IgM (green histogram) and IgG (blue histogram), and less strongly to IgA (brown histogram). This experiment demonstrates that bacteria from the intestine of rabbits can bind to intact Igs. These data support the idea that in the FAE, bacteria or bacterial products may bind to B cells through surface Ig.

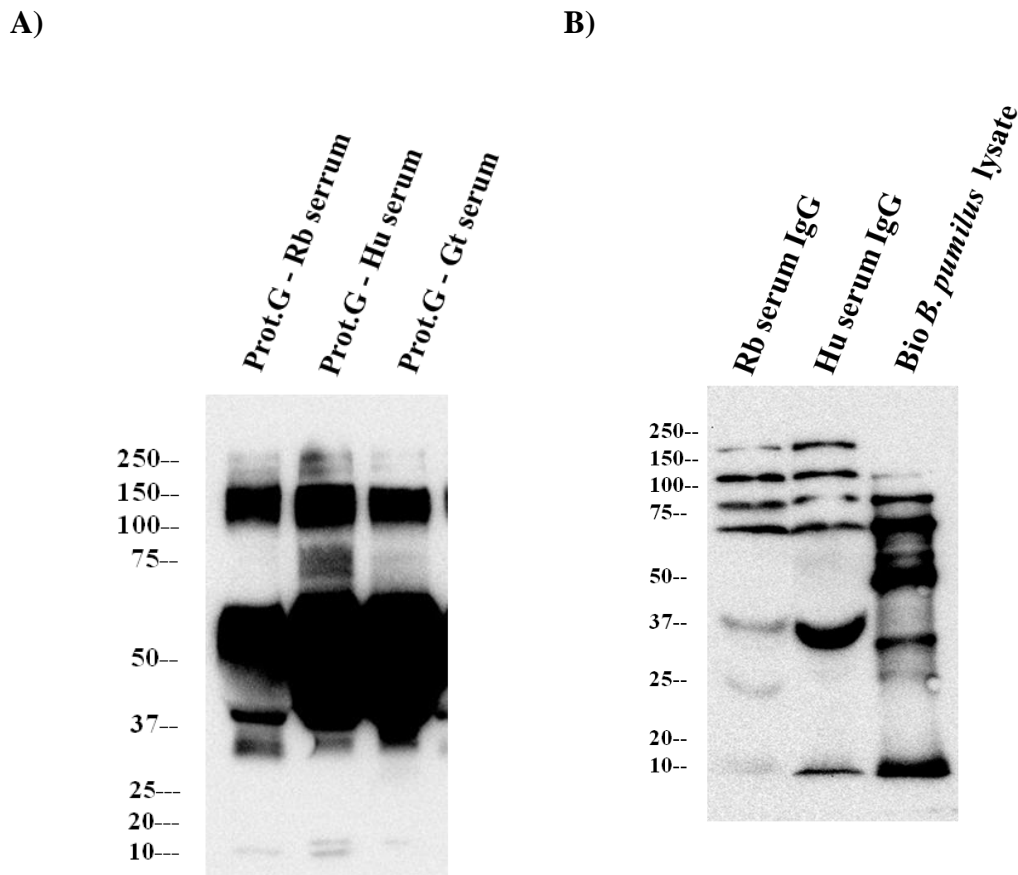


Figure 14: Western blot analysis of *B. pumilus* surface molecules

immunoprecipitated with serum IgG. *B. pumilus* vegetative cells were biotinylated with 2mg/mL EZ-Link Sulfo-NHS-LC-Biotin. The cells were lysed in lysis buffer (1.5M NaCl; 1% NP-40; 1% Triton X-100). The biotinylated bacterial molecules were immunoprecipitated with rabbit, human or goat serum IgG conjugated to Protein G beads. The proteins were detected with HRP conjugated Streptavidin.

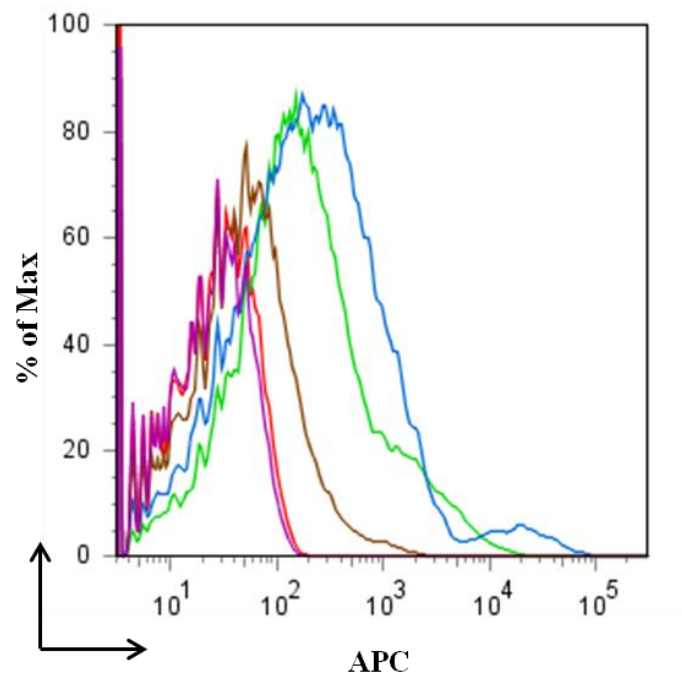


Figure 15: Flow cytometric analysis of *B. pumilus* binding to rabbit serum Ig.

B. pumilus vegetative cells were incubated with normal rabbit serum. The cells were stained with biotinylated 367 anti-IgM mAb (1:200) (green), 1 µg/mL biotinylated anti-Rb IgA (brown), or 3.9 µg/mL biotinylated goat α-rabbit Fcγ (blue). Binding activity was detected with Streptavidin-conjugated APC.

B cell expression of chemokine receptors

B cells that enter GALT follow a chemokine gradient to the FAE where they may encounter bacteria. These events likely provide the B cells with signals that may lead to their activation and relocation to other regions of the follicle. To determine whether bacteria provide such signals, I stimulated stable rabbit B cell lines PBL-1, 55D1 and 79E for a period of 4 hours through the BCR (goat anti-rabbit IgM) or through TLR2 (TLR agonist, Pam3Cys4). These three cell lines are at different stages of B cell development: PBL-1 cells are at a pre-B cell stage (IgM^{Lo} B cells); 55D1 are immature B cells (IgM^{Hi} B cells); and 79E are cells that have gone through a germinal center reaction and have somatically diversified *VDJ* IgH genes (Sethupathi, *et al.*, 1994). If the stimulation of B cells through these receptors provides signals that induce their migration, then they should express specific chemokine receptors on their surface. Since CCR6, CXCR5, CCR7 and CXCR4 are expressed on B cells as they move throughout the follicle in GALT (Hanson and Lanning, 2008; Knight Lab working model), I decided to test their expression on the stimulated B cells.

I found that that the expression of all four chemokine receptors CCR6, CXCR5, CCR7 and CXCR4, is up-regulated upon TLR2 stimulation (green histogram) in PBL-1 cells compared to unstimulated cells (red histogram). In contrast, the cells that were stimulated through the BCR (blue histograms) did not express these chemokine receptors relative to the unstimulated control (Figure 16A-D).

The detection of chemokine receptor expression on 55D1 cells is shown in Figure 17. I observed that upon TLR2 stimulation (green histogram), 55D1 expressed CCR6 (Fig. 17A), but not CXCR5, CCR7 or CXCR4 (Fig. 17B-D). Interestingly, when the cells were stimulated through the BCR (blue histogram) some chemokine receptors were down-regulated: CXCR5, CCR7 and partially CCR6 (fig. 17A-C). CXCR4 was not affected by stimulation through either receptor (Fig. 17D).

The same process of stimulation applied to PBL-1 and 55D1 B cells was used on 79E cells. I observed a significant up-regulation of CCR6 upon stimulation through TLR2 compared to the unstained control (Fig. 18A); CCR7 and CXCR4 were slightly up-regulated as well (Fig. 18, C&D). Only CCR7 was slightly up-regulated upon stimulation through BCR (Fig. 18C); the other chemokine receptors were not affected by stimulation through BCR (Fig. 18A, B&D).

Overall, the results from these experiments suggest that stimulation through TLR2 on stable rabbit B cells provide signals that induce the activation of chemokine receptors. These data support the idea that bacteria provide B cells with signals that may lead to their migration to other regions of the GALT follicles.

B cell activation in vitro

B cell activation is initiated following the recognition of antigen through the BCR and results in B cell proliferation and differentiation. The binding of specific antigen to the BCR initiates intracellular signaling and results in B cell activation (Harwood and Batista, 2010). TLR signaling, which occurs by bacterial molecules, is also involved in

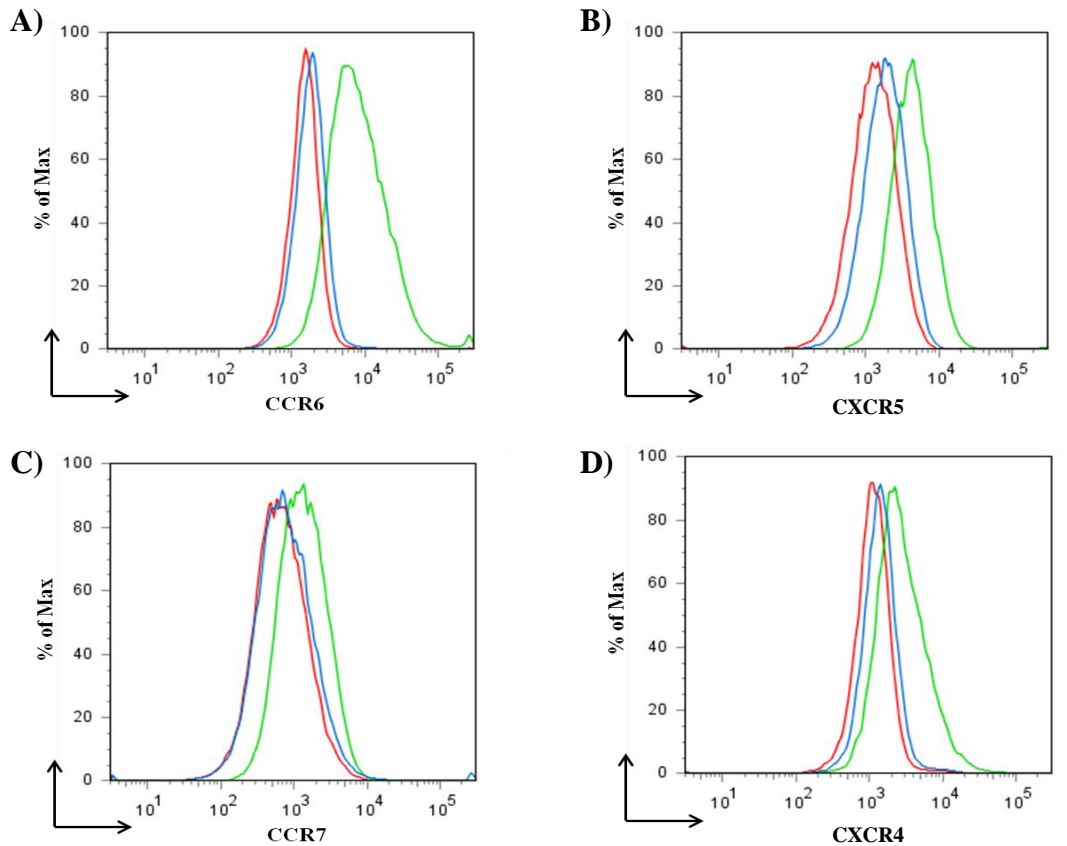


Figure 16: Flow cytometric analysis of chemokine receptor expression on PBL-1 cells. Rabbit B cells PBL-1 were stimulated for 4 hours with goat anti-rabbit IgM (1:10) (blue), with 10 μ g/mL TLR2,6 agonist Pam3Cys2 (green), or left unstimulated (red). The cells were stained with A) PECy5.5-conjugated RbCCL20-Ig against CCR6, B) APC-conjugated anti-HuCXCR5, C) PE-conjugated anti-HuCCR7, and D) PECy7-conjugated anti-HuCXCR4. The analysis was performed in a CantoII flow cytometer.

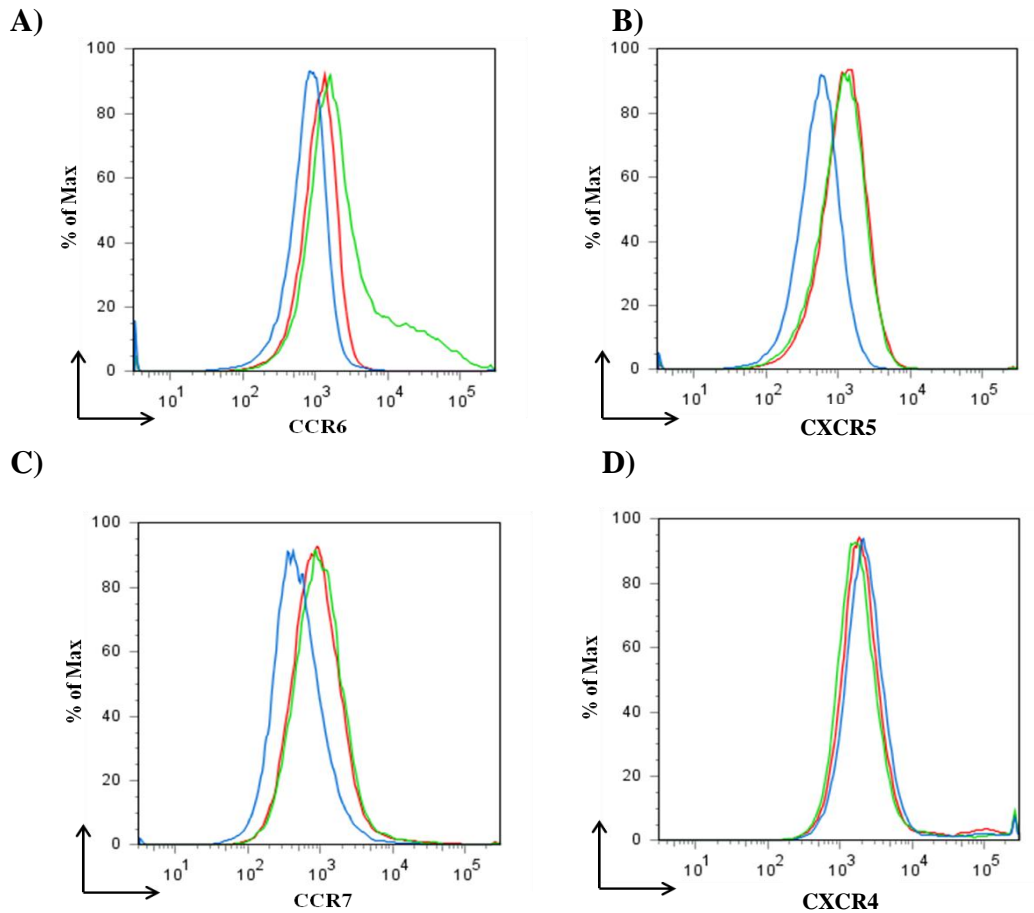


Figure 17: Flow cytometric analysis of chemokine receptor expression on 55D1 cells. Rabbit B cells 55D1 were stimulated for 4 hours with goat anti-rabbit IgM (1:10) (blue), with 10 μ g/mL TLR2,6 agonist Pam3Cys2 (green), or left unstimulated (red). The cells were stained with A) PECy5.5-conjugated RbCCL20-Ig against CCR6, B) APC-conjugated anti-HuCXCR5, C) PE-conjugated anti-HuCCR7, and D) PECy7-conjugated anti-HuCXCR4. The analysis was performed in a CantoII flow cytometer.

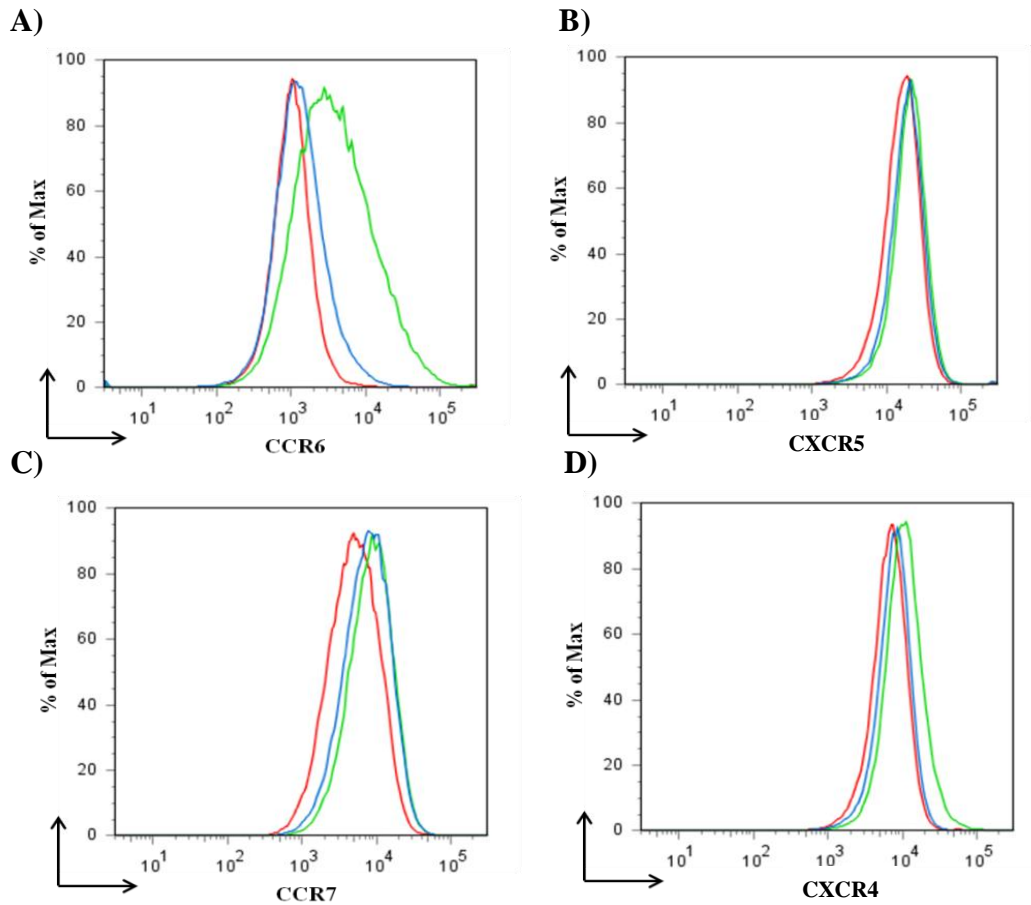


Figure 18: Flow cytometric analysis of chemokine receptor expression on 79E

cells. Rabbit B cells 79E were stimulated for 4 hours with goat anti-rabbit IgM (1:10) (blue), with 10 μ g/mL TLR2,6 agonist Pam3Cys2 (green), or left unstimulated (red). The cells were stained with A) PE-Cy5.5-conjugated RbCCL20-Ig against CCR6, B) APC-conjugated anti-HuCXCR5, C) PE-conjugated anti-HuCCR7, and D) PE-Cy7-conjugated anti-HuCXCR4. The analysis was performed in a CantoII flow cytometer.

multiple stages of B cell activation and differentiation (Pasare and Medzhitov, 2005). All TLR signaling pathways culminate in activation of the transcription factor nuclear factor-kappaB (NF- κ B), which controls the expression of an array of inflammatory cytokine genes (Kawai and Akira, 2007). In the following series of experiments, I tested if bacterial molecules activate B cells by testing for activation of NF- κ B, expression of the activation marker, B7, and for altered expression of chemokine receptors.

NF- κ B-Luciferase detection in activated B cells

To determine if bacterial molecules stimulate B cell activation *in vitro*, I transfected rabbit B cell lines PBL-1, 55D1 and 79E with an NF κ B-Luciferase reporter vector (5kb). In addition, the cells were co-transfected with pCEP4 (10kb) because the Luciferase reported construct did not have a selectable marker for mammalian cells. PCEP4 contains a hygromycin selection gene for mammalian cells, and I used hygromycin to select stable transfectants. Genomic DNA was isolated from stable transfectants and PCR was performed to check for the presence of the Luciferase gene.

By PCR analysis, I did not find a band corresponding to the Luciferase (*luc*) gene (169bp in size). I repeated these transfections in two additional experiments, and each time I did not find the *luc* gene; consequently, I abandoned this approach. A representative experiment is depicted in Figure 19. The expected size band (169bp) indicating that the cells contained the Luciferase gene was not observed in neither 79E cells (Fig. 19A), nor 55D1 cells (Fig 19B).

It is possible that the assay did not work because instead of using an excess of NF κ B-Luciferase vector over pCEP4 to enhance the chances of obtaining transfectants, I used equal molar amounts of pCEP4 and NF κ B-Luciferase.

Expression of B7 on activated B cells

B7 is normally up-regulated on B cells following activation by cytokines, or ligation of cell surface molecules (e.g. MHC class II and CD40). In addition, B7 can be rapidly induced on B cells following activation by cross-linking the BCR (Jirapongsananuruk, 1999).

As another means of testing if rabbit B cells can be activated by bacterial molecules, I stimulated B cells and tested for the expression of B7. I stimulated the stable rabbit B cell line 55D1 *in vitro* for 4 hours with sonicated *B. pumilus*, with purified exopolysaccharide (eps) from *Bacillus subtilis* (sinRtasA), or with a similar polysaccharide preparation derived from a *B. subtilis* mutant (sinRtasAepsH). To assess for B cell activation I detected the expression of B7, present on activated B cells but not on resting B cells. Since B7 is the ligand for CTLA-4 (Linsley, 1991), a T cell surface molecule, I used biotinylated CTLA4-Ig to detect the expression of B7. The stimulated cells were analyzed by flow cytometry.

My expectation was that if bacterial molecules can activate B cells, then I would find up-regulation of B7 on the surface of 55D1 cells after exposure to bacterial molecules. The histograms depicted in Figure 20 are the result of two independent experiments. The first experiment showed that B7 is up-regulated upon stimulation with

the eps from *B. subtilis* (green histogram) but not when stimulated with the polysaccharide preparation of the mutant (red histogram) (Fig. 20A). I also found that sonicated *B. pumilus* induced the up-regulation of B7 (green histogram), compared to the unstimulated control (red histogram) (Fig. 20B).

In a second experiment, I confirmed that *B. subtilis* eps stimulated the expression or up-regulation of B7 (Fig. 20C, green histogram) compared to the unstimulated control (red histogram). However, I also found that the mutant polysaccharide preparation also stimulated the expression of B7 (Fig. 20D, green histogram).

The results of this experiment suggested that, exopolysaccharides from *B. subtilis* as well as *B. pumilus* surface molecules can activate 55D1 B cells. I repeated this experiment twice and found that in addition to the eps, other unidentified *B. subtilis* polysaccharide molecule(s), found in the mutant preparation, can also induce the up-regulation of B7.

Expression of chemokine receptors on activated B cells

The results obtained above suggest that rabbit B cells can be activated by bacterial molecules (e.g. eps). In addition, I have demonstrated that stimulation through TRL2 using a TLR agonist induces the expression or up-regulation of chemokine receptors. These two findings support the idea that in the rabbit GALT, bacteria can provide B cells with signals that may lead to their activation and relocation to other regions of the follicle.

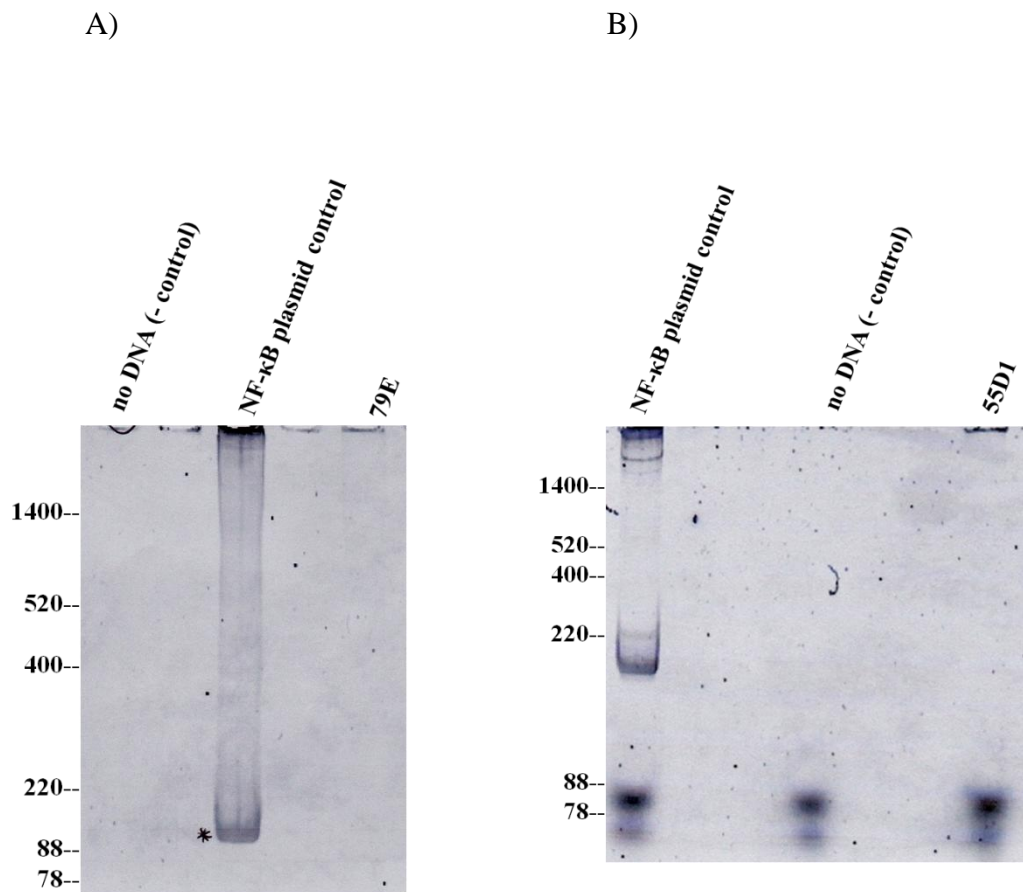


Figure 19: PAGE of PCR-amplified Luciferase gene from transfected rabbit B cells. A) 79E cells and B) 55D1 cells were transfected with 10 μ g pNF- κ B-Luc and 4 μ g pCEP4. Genomic DNA was extracted from each cell type and PCR amplified using Luciferase internal primers. pNF- κ B was the positive control, and the negative control was without genomic DNA. The expected product size was 169bp.

To determine whether bacteria can provide such signals, I stimulated stable rabbit B cell lines PBL-1 and 55D1 for a period of 4 hours with sonicated *B. pumilus*, *B. subtilis* eps, and the mutant strain polysaccharide preparation. These molecules can activate B cells (Fig. 20). If the activation of B cells is linked to the expression of molecules involved in migration, then I would expect to detect the up-regulation of chemokine receptors CCR6, CXCR5, CCR7 and CXCR4 upon B cell activation. These receptors are expressed on B cells as they move throughout the follicle in GALT.

I observed that PBL-1 cells did not up-regulate CCR6, CXCR5 and CCR7 (Fig. 21A-C, left) upon stimulation with sonicated *B. pumilus* (green histogram), eps (blue histogram) or the mutant polysaccharide (brown histogram). However, CXCR4 was slightly up-regulated upon sonicated *B. pumilus* stimulation (Fig. 21D, LEFT, green histogram) compared to unstimulated cells (red histogram). CXCR4 was also up-regulated to a lesser extent upon stimulation with eps or the mutant preparation (Fig. 21, E&F, LEFT, green histograms). Stimulation through BCR (green histogram) and TLR2 (blue histogram) (Fig. 21A-D, RIGHT) confirmed the results shown in Figure 16.

Similar results were obtained with stimulated 55D1 B cells. Once again CCR6, CXCR5 and CCR7 were not up-regulated upon stimulation with bacterial molecules (Fig. 22A-C) compared to unstimulated control (red histogram). Only CXCR4 appeared to be slightly up-regulated upon stimulation with sonicated *B. pumilus* (green histogram), eps (blue histogram) and the mutant polysaccharide preparation (brown histogram).

Stimulation through BCR and TLR2 (data not shown) confirmed the results shown in Figure 16.

The data provided by these experiments do not explain if the activation of B cells is linked to the expression of molecules involved in migration, such as chemokine receptors CCR6, CXCR5, CCR7 and CXCR4.

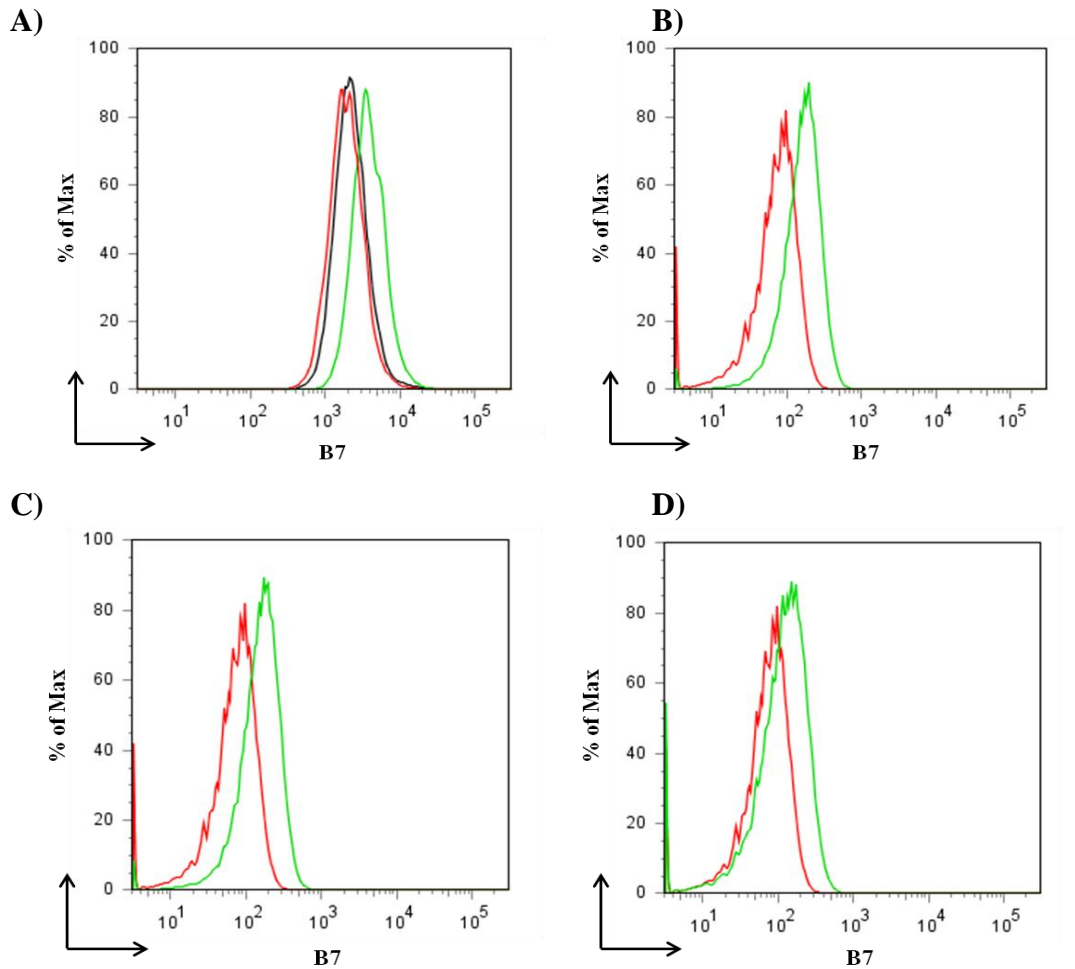
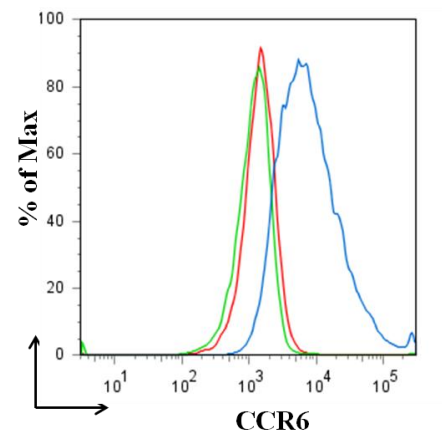
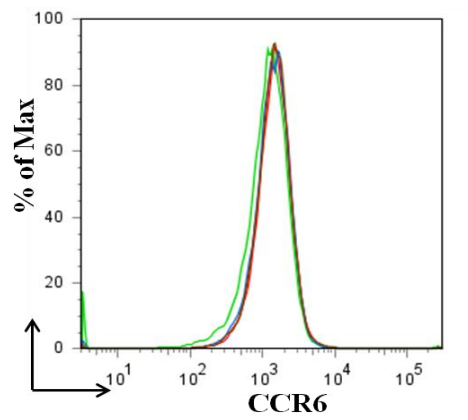
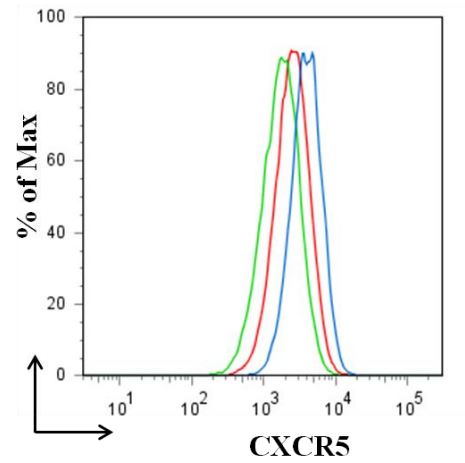
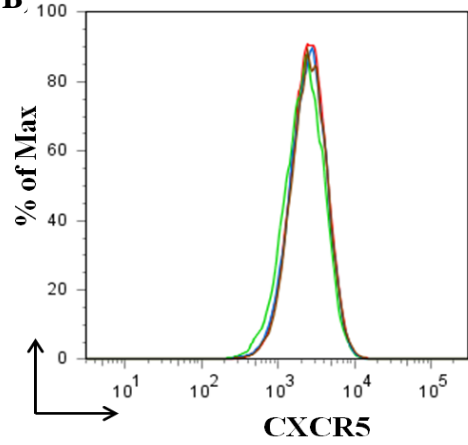


Figure 20: Flow cytometric analysis of B7 expression on 55D1 cells. Rabbit B cells 55D1 were stimulated for 4 hours with A&C) 2.5µg/mL eps from *Bacillus subtilis*, or with D) *B. subtilis* eps null mutant preparation, or with B) 200µL sonicated *Bacillus pumilus*. The cells were stained with biotinylated CTLA4-Ig, followed by APC-conjugated Streptavidin. The cells were analyzed in the CantoII cytometer.

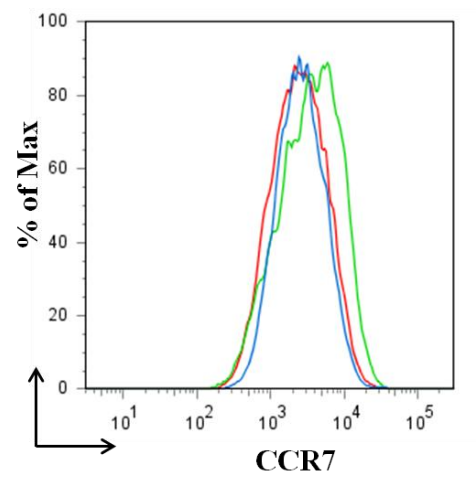
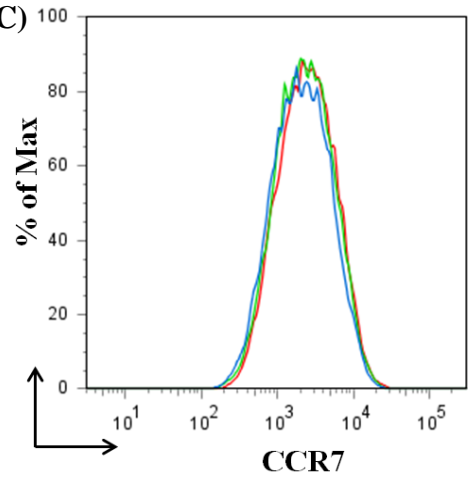
A)



B)



C)



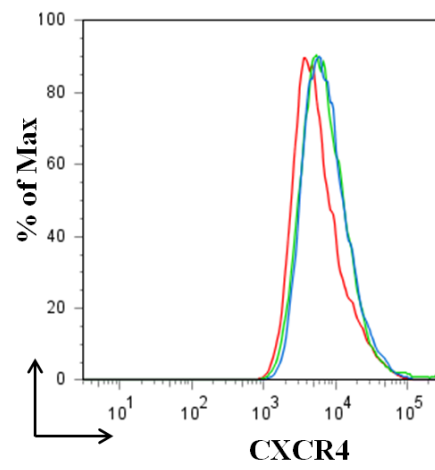
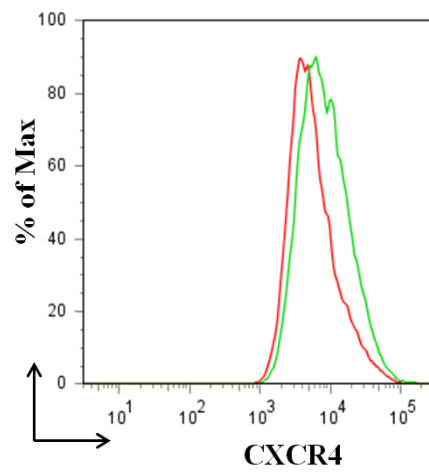
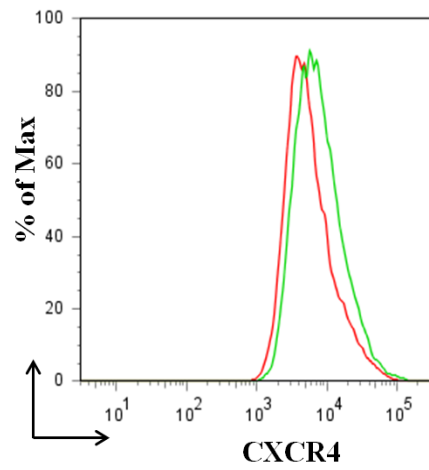
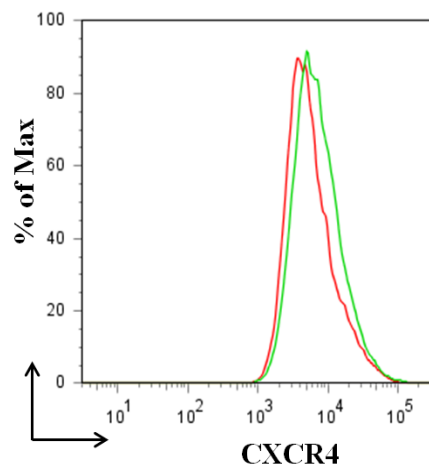
D)**E)****F)**

Figure 21: Flow cytometric analysis of chemokine receptor expression on activated B cells. Rabbit B cells PBL-1 were stimulated for 4 hours with LEFT SIDE: sonicated *B. pumilus* (green), 2.5µg/mL eps from *Bacillus subtilis* (blue), 2.5µg/mL *B. subtilis* eps null mutant preparation (brown), or left unstimulated (red); RIGHT SIDE: goat anti-rabbit IgM (1:10) (green), with 10µg/mL TLR2,6 agonist Pam3Cys2 (blue), or left unstimulated (red). The cells were stained with A) PECy5.5-conjugated RbCCL20-Ig against CCR6, B) APC-conjugated anti-HuCXCR5, C) PE-conjugated anti-HuCCR7, and D-F) PECy7-conjugated anti-HuCXCR4. The analysis was performed in a CantoII flow cytometer.

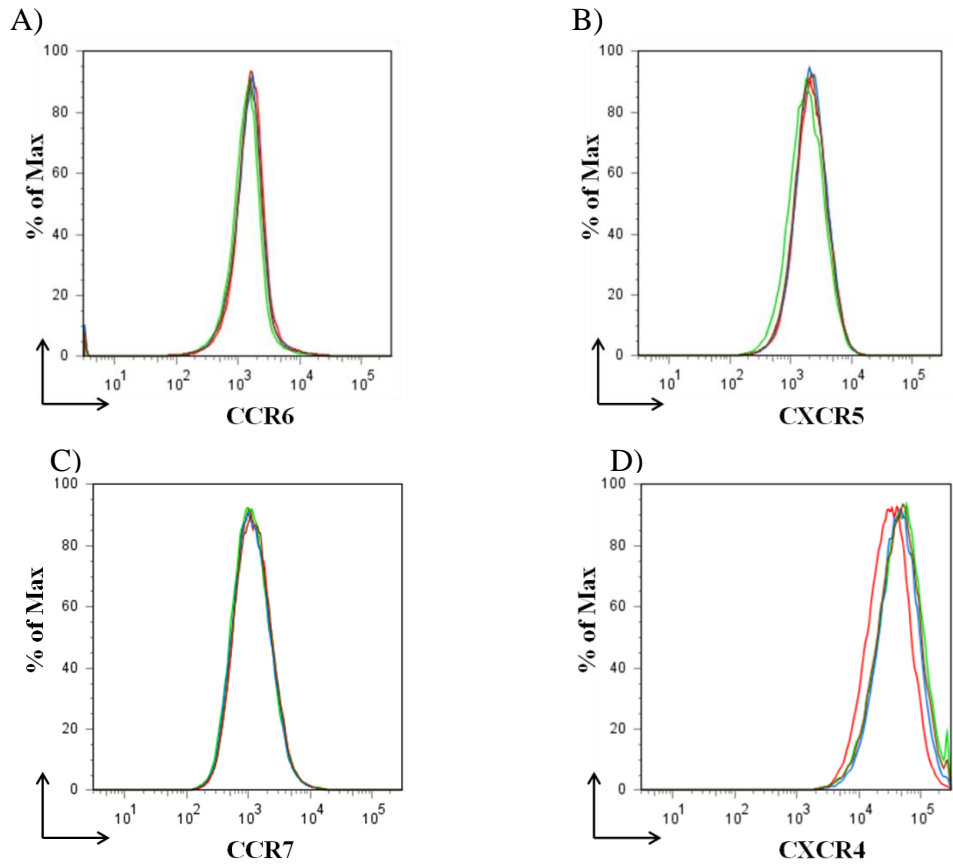


Figure 22: Flow cytometric analysis of chemokine receptor expression on activated B cells. Rabbit B cells 55D1 were stimulated for 4 hours with sonicated *B. pumilus* (green), 2.5 μg/mL eps from *Bacillus subtilis* (blue), 2.5 μg/mL *B. subtilis* eps null mutant preparation (brown), or left unstimulated (red). The cells were stained with A) PECy5.5-conjugated RbCCL20-Ig against CCR6, B) APC-conjugated anti-HuCXCR5, C) PE-conjugated anti-HuCCR7, and D) PECy7-conjugated anti-HuCXCR4. The analysis was performed in a CantoII flow cytometer.

CHAPTER FOUR

DISCUSSION

The intestinal microbiota has a role in the generation and diversification of B cells in the GALT of rabbits. The interactions between the host and intestinal microbiota alter the B cell repertoire in an antigen- and T cell-independent manner. In the process of elucidating the mechanism by which the above mentioned processes occur, I isolated and identified bacterial species from the appendix of rabbits that bind to B cell surface Ig. Using FvIg recombinant molecules I successfully detected a ~20kDa molecule from the isolated intestinal bacteria. I also showed that molecules from these bacterial species bind to rabbit serum Ig. My *in vitro* experiments collected evidence that suggest that bacterial molecules provide signals through BCR, TLR2 or both, that stimulate the activation of rabbit B cells. By performing stimulation experiments, I found that stimulation through TLR2 alters the expression of chemokine receptors on the surface of B cells; and that their expression varies with the stage of development of the cell.

These findings provide insights into the mechanism by which the microbiota provide rabbit B cells with signals that may lead to their activation and relocation to other regions of the follicle in GALT. In the following sections, I will discuss how these findings contribute to the current model of B cell development and expansion.

Intestinal bacterial molecules bind Ig

B cell expansion and the somatic diversification of Ig genes in the GALT of rabbits occur in an antigen- and T cell-independent mechanism (Pospisil and Mage, 1998; Yeramilli and Knight, 2010). The process of somatic diversification requires interaction of GALT with intestinal microbiota (Rhee, *et al.*, 2004). At least one study suggests that B cells can be polyclonally stimulated *in vitro* by bacteria through the engagement of IgM by B cell superantigens (Severson, 2010).

The expansion of B cells and somatic diversification of Ig genes is polyclonal and seems to occur independent of specific antigen. This led us initially to hypothesize that bacteria express a superantigen-like molecule that binds to the V_H and/or V_L region of IgM on the surface of rabbit B cells, independent of their antigen specificity, triggering the expansion of B cells in GALT. Since in *Alicia* rabbits the expansion of V_{HA} allotype over V_{HN} allotype B cells occurs upon contact with microbiota (Rhee, *et al.*, 2004), we also hypothesized that the putative bacterial superantigen binds to V_{HA} B cells but not V_{HN} B cells in GALT.

With this idea in mind, I isolated three species of interest, *B. pumilus*, *B. thuringiensis* and *P. acnes*, from the appendix of rabbit using a⁺FvIg (V_{HA}) and a⁻FvIg (V_{HN}) molecules. I performed a series of experiments to validate the initial hypothesis, including re-screening experiments by flow cytometry to confirm the results obtained by FACS sorting; however, the obtained results were very inconsistent.

I found that the condition of the culture (e.g. liquid culture versus agar plate culture), as well as the maturity of the culture influenced whether the isolates bound to V_{Ha} or V_{Hn} allotypes (FvIg). It is possible that the inconsistent binding to FvIg was due to transient expression of the bacterial molecule(s) from the isolated species. Perhaps such molecule(s) is only expressed at specific stages during bacterial growth. This can be assessed by obtaining time-point samples of a growing culture, staining them with FvIg and performing flow cytometry experiments to determine if there is a difference in binding activity to FvIg. It is also possible that the nutrients present in the growth media influence the metabolism of the specie, altering the expression of the putative bacterial molecule(s). A logical approach to solve this enigma is to expose the isolates to different culture conditions and perform binding experiments to determine the optimal condition where bacteria express such molecule(s).

During these experiments, I successfully identified a ~20kDa bacterial molecule from the surface of *B. pumilus*, *B. thuringiensis* and *P. acnes*, that immunoprecipitated with both a⁺FvIg and a⁻FvIg. These data indicated that bacterial molecules can bind Ig, and supported the idea that in the FAE, bacteria provide B cells with signals that may lead to their activation. On the other hand, these results also led us to speculate that the inconsistent binding to FvIg observed by flow cytometry experiments were due to transient expression of this ~20kDa molecule. This question can be answered by growing the isolates under different conditions and performing immunoprecipitation experiments in each case using FvIg molecules. This will allow determining if the ~20kDa molecule

is transiently expressed on these species, and if it can be responsible for the inconsistent results observed by the flow cytometric experiments.

I also found that *B. pumilus* molecules, other than the ~20kDa molecule immunoprecipitated by FvIg, bound directly to different regions of serum IgG from rabbit, human or goat species. In addition, I observed that the same bacterial molecules did not bind to the same sites on the IgG from these three animals. These observations not only confirm that Ig molecules from different species are unique to that species, but also suggest that the same bacteria can bind to different regions of IgG, depending on the species. These results provide further evidence that bacterial molecules can bind Ig. This finding is consistent with the hypothesis that in the FAE, bacterial molecules may bind to B cells through surface Ig, providing signals that may stimulate B cell activation.

Newborn *Alicia* rabbits possess a predominant V_{Hn} B cell population, but as these rabbits age, the number of V_{Ha} B cells increases, becoming the predominant allotype (Pospisil and Mage, 1998; Zhu, 1999). Their V_{Ha} B cell population lacks the IgH V_H gene segment, *V_HI*. Instead, the majority of the VDJ rearrangements in these rabbits utilize the functional *V_H4* gene (Zhu, 1999). Because the expansion of V_{Ha} B cells occurs with age and upon contact with bacteria (Rhee, *et al.*, 2004), it is apparent that the *ali/ali* rabbits offer a unique opportunity to understand the selective forces brought to the immune system by the intestinal microbiota.

One of my goals during this study was to test if bacteria from the intestine of *Alicia* rabbits interact with the Ig of V_{Ha} but not V_{Hn} B cells, thereby inducing the expansion of V_{Ha} B cells. My strategy was to substitute the *V_HI* gene present in the FvIg

constructs (Severson, 2010) by the V_H4 gene which is used in many B cells of *ali/ali* rabbits. During my cloning experiments I successfully obtained the correct sequence of a complete FvIg molecule containing the V_H4 gene. However, after further testing I found that the FvIg protein was not expressed by the transfected mammalian CHO cells (see Chapter 2, Materials and Methods). Although I revised and adjusted my cloning strategy several times, I was unable to achieve complete expression of the FvIg molecules, and I abandoned this plan.

Previously, I successfully demonstrated that bacterial molecules can bind Ig, supporting the idea that in the FAE, bacteria provide B cells with signals that may lead to their activation. As a consequence, I became interested in investigating the mechanism by which commensals drive GALT reactions, particularly the processes that lead to the activation and migration of B cells in GALT.

B cell activation and expression of B7

The activation of B cells can occur by BCR crosslinking (Harwood and Batista, 2010); through TLR stimulation (Pasare and Medzhitov, 2005); or by the combination of stimuli through more than one surface receptor, including CD40. Previous studies in the Knight Lab tested B cell activation in rabbit by various methods, including Calcium flux. The alteration in intracellular calcium ions (Ca^{2+}) is one of the most rapid cellular responses to a variety of stimuli, and suggests cell activation. However, these experiments provided inconclusive data.

One of the most distinctive phenotypes of activated B cells is the surface expression of B7 (Jirapongsananuruk and Leung, 1999). I reasoned that the detection of B7 would be a direct and effective method for the recognition of activated B cells. The first problem I encountered while carrying out these experiments was the unavailability of antibodies against rabbit B7. I solved this problem by using soluble receptor, biotinylated rabbit CTLA4-Ig to detect the expression of B7. The T cell surface molecule CTLA4 is a receptor for B7 (Linsley, 1991).

I chose to use the stable rabbit cell line 55D1 to determine if bacterial molecules provide signals that may induce the activation of B cells. Other stable cell lines, such as PBL-1 and 79E, were less favored choices since they are at an early stage of development (PBL-1) and at a later one (79E) (Sethupathi, *et al.*, 1994).

I found that exopolysaccharide from *B. subtilis*, as well as *B. pumilus* surface molecules can activate 55D1 rabbit B cells. These findings support the idea that B cells can interact with specific bacterial molecules in the FAE of the follicle; and that this interaction may provide signals that induce the activation of B cells.

One of the caveats of stimulating cells with sonicated bacteria (*B. pumilus*) was that I had no means to determine the identity of the molecule(s) inducing the activation of 55D1 cells. In addition, I could not estimate the concentration of the molecule(s) involved in the stimulation of the cells. One way to solve this problem is to identify by mass spectrometry the ~20kDa detected by FvIg molecules. Once identified, the protein could be purified and used for stimulation experiments. Although I demonstrated that this molecule binds to Ig molecules, it is possible that it can also bind to other receptors

on the surface of B cells, such as TLRs. These questions remain to be answered by future studies.

The fact that at least one stimulus (*B. subtilis* eps) is a known purified molecule, was quite exciting because it provided the opportunity to ask a new set of questions. However, I found later that *B. subtilis* polysaccharide(s), the negative control, was also able to stimulate the activation of B cells. One possibility is that eps and polysaccharide molecule(s) stimulate B cells through distinct receptors, and that the end result for both stimuli is the same, the activation of B cells. Other studies in the Knight Lab are currently trying to determine the specific receptor for *B. subtilis* eps. Some clues indicate that such receptor may be TLR2, at least in mice; however, all results are inconclusive thus far. Finding the receptor for eps would allow for a more extensive understanding of the mechanism by which bacterial molecules influence B cell expansion. Future experiments could involve knockout rabbits lacking the putative receptor. This would provide a tool to ask how this condition can affect B cell expansion and diversification in rabbits.

During these studies, I also found that 55D1 cells were activated when stimulated with goat anti-rabbit IgM, but only in the absence of CD40L. This is not a surprising result in view of the fact that activation of B cells can occur by BCR crosslinking (Harwood and Batista, 2010). Additionally I found that soluble CD40L down-regulates the expression of B7. It is possible that soluble CD40L was endocytosed by 55D1, triggering some internal signaling that inhibited activation of B cells and by association,

the expression of surface B7. It is also possible that the activation of the cells was not affected, but for some unexplainable reason, the expression of B7 was inhibited.

B cell expression of chemokine receptors

The Knight Lab has gathered evidence over the past decades to support the idea that bacteria from the intestines of rabbits are required for the expansion and migration of B cells in GALT. A portion of this compilation of evidence has been converted into a simplified working model (Fig. 23) that proposes the mechanism by which B cells migrate throughout the follicle. This model suggests that B cells that enter the follicle follow a chemokine gradient that starts at the FAE, and concludes at the basolateral end of the follicle, where cell proliferation and Ig diversification occur.

Most of the results contributing to this model were obtained by *in situ* hybridization studies (Hanson and Lanning, 2008), which provide the exact location of chemokine expression in the follicle. These chemoattractants are CCL20, CXCL13, CCL21 and CXCL12. Flow cytometric experiments in which primary B cells from the appendix of rabbits are stained with antibodies against chemokine receptors demonstrate that these cells express CCR6, CXCR5, CCR7 and CXCR4 receptors (unpublished results). These are the receptors for the ligands detected by *in situ* hybridization experiments, respectively.

One of the obstacles encountered when working with primary cells from the appendix of young rabbits is that the isolated B cells die at a fast rate. In addition to this, it is difficult to obtain a homogeneous B cell population from a tissue so rich in other cell

types (e.g. epithelial cells and stromal cells). To overcome this obstacle, I tested stable rabbit B cell lines with the idea of generating an *in vitro* system that would facilitate asking questions about B cell migration in GALT.

Since our model suggests that B cells leave the BM marrow and migrate to GALT, I reasoned that PBL-1, an early stage B cell, would successfully mimic these resting B cells from the BM. In our model, naïve B cells migrate to the FAE and become activated by bacterial molecules. To better study the fate of these activated B cells in the follicle, I decided to test 55D1 cells, a more mature cell line. Finally, I needed a cell line that had already gone through the diversification of *V(D)J* genes. This cell line (79E) would be used to study the cells found in the basolateral end of the follicle.

One of the major difficulties encountered when working with rabbits is the lack of antibody reagents against molecules of this species. I was able to circumvent this issue by using human antibodies that react against rabbit protein, generously provided by Dr. Dennis Lanning.

I found that the expression of all four chemokine receptors CCR6, CXCR5, CCR7 and CXCR4, is up-regulated upon TLR2 stimulation in PBL-1 cells. TLR2 recognizes bacterial lipoproteins and peptidoglycans (Forchielli, 2005). One of the major caveats to this experiment, and to other experiments with 55D1 and 79E cells, is that instead of using bacterial molecules to stimulate the cells, I used the TLR2,6 agonist, Pam3CSK4. Nevertheless, I demonstrated that early stage PBL-1 cells can express all four chemokine receptors detected in primary cells from the appendix (Hanson and Lanning, 2008; Knight Lab working model). I also stimulated PBL-1 for 24 hours with

the same reagents, but the results were comparable to the results I obtained by stimulating the cells for 4 hours. This suggests that the expression of chemokine receptors in stimulated cells is a stable rather than a transient event. The cells were also stimulated in the presence of CD40L, since CD40 is essential in B cell activation (Pasare and Medzhitov, 2005). However, whether CD40 was present or absent, the results were the same (data not shown). PBL-1 cells were also stimulated with LPS (against TLR4) and the TLR1,2 agonist Pam2CSK4, but the results did not differ from the unstimulated control (data not shown).

The stimulation of 55D1 and 79E cells through TLR2 resulted in the up-regulation of CCR6 but not CXCR5, CCR7 and CXCR4. This was more apparent in 79E cells. Once again, these cells were stimulated in the presence of CD40L, but this factor did not make a difference in the expression of chemokine receptor in these cells. Stimulation with LPS and Pam2CSK4 was comparable to the unstimulated control.

In summary, these data suggested that TLR2 stimulation alter the chemokine expression of B cells, and may be important in the migration of B cells in GALT. It is not surprising that LPS did not stimulate PBL-1, 55D1 or 79E cells, since it has been known for many years that rabbit B cells do not react to this bacterial molecule. The finding that Pam2CSK did not stimulate the expression of any of the four chemokine receptors suggests that TLR1 may not contribute to the migration of B cells in GALT.

Stimulation through BCR appears to play a lesser role than TLR2 in this process, as evidenced by the downregulation of CXCR5, CCR6 and CCR7 in 55D1cells but not in PBL-1 and 79E cells. It was not completely surprising that PBL-1 cells did not express

any of the chemokine receptors upon BCR stimulation, given that these cells are IgM^{Lo} B cells (Sethupathi, *et al.*, 1994).

The different levels of expression of chemokine receptors in the three B cell lines might be due to their stage of development, and not completely on the nature of the stimuli. Another possibility is that B cells leaving the BM may initially have the capacity to upregulate all four chemokine receptors upon stimuli, but the expression of selected receptors is lost after receiving a variety of peripheral signals.

The data collected by these *in vitro* studies (Table 3) suggest that stimulation through TLR2 on rabbit B cells provide signals that induce the activation of some chemokine receptors. These findings further support the idea that bacteria provide B cells with signals that may lead to their activation and movement to other regions of the GALT follicles.

B cells leave the BM and enter GALT two days after birth. Previous studies in the Knight Lab have demonstrated that a⁺FvIg but not a⁻FvIg binds M cells in the FAE, suggesting that early in development, the expansion of V_Ha B cells but not V_Hn B cells occurs by events taking place in the FAE. However, in later experiments, proliferating V_Hn B cells were detected in the basolateral end of the follicle, contradicting their initial observation. These findings suggested that the initial activation of B cells in the early stages of the GALT response do not require migration to the FAE; and that the selective expansion of B cells can occur after B cell proliferation and somatic diversification of Ig genes. According to these findings, it is possible that somatically diversified B cells traffic back to the FAE where bacterial molecules provide B cells with signals that lead to

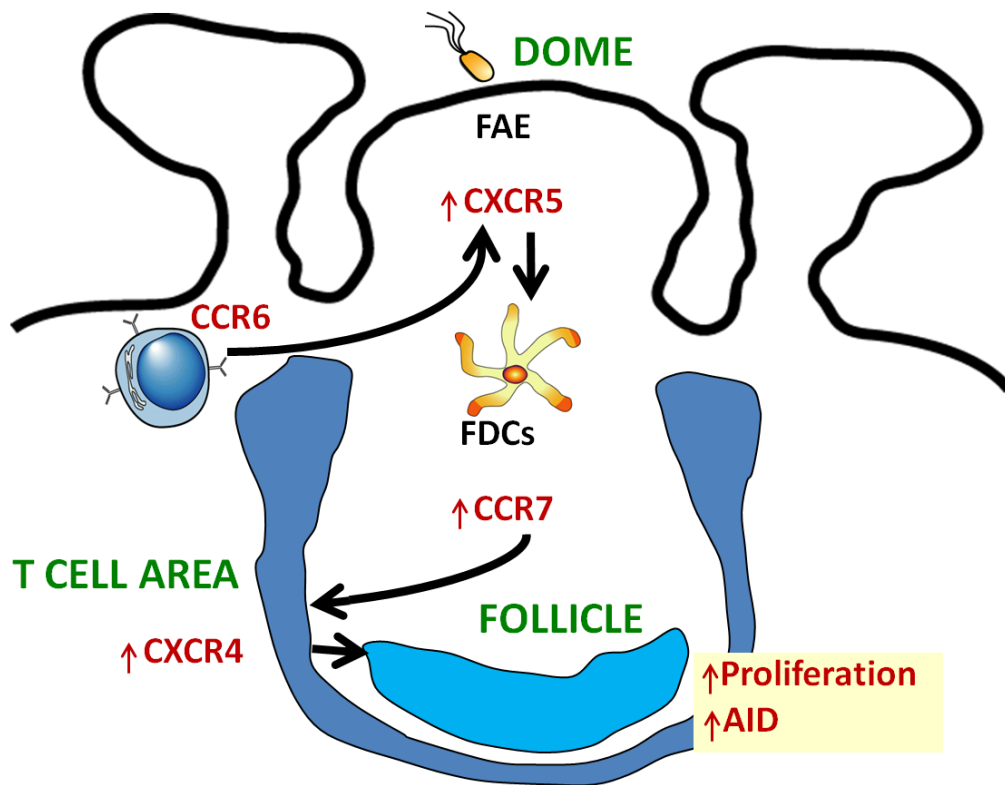


Figure 23: Knight Lab model of B cell migration in GALT. Resting B cells leave the BM approximately 2 days after birth and enter GALT. Once in GALT, B cells expressing CCR6 follow a chemokine gradient to the FAE (dome), where CCL20 is produced by epithelial cells. In this region, B cells may encounter bacterial molecules which provide B cells with signals for migration. The cells express CXCR5 and migrate next to the FDC zone, where CXCL13 is produced. B cells receive extra stimulatory signals from FDC, upregulate CCR7, and migrate to the T cell areas, attracted to CCL21. Finally, B cells expressing CXCR4 migrate to the basolateral end of the follicular area, where CXCL12 is expressed. In this area, the cells proliferate and the Ig genes undergo somatic diversification.

Table 3: Summary of chemokine receptor expression on rabbit B cell lines

	Stimuli	PBL-1	55D1	79E
CCR6	BCR	NC*	-	NC
	TLR2	+++	+	+++
CXCR5	BCR	NC	---	NC
	TLR2	++	NC	NC
CCR7	BCR	NC	---	-
	TLR2	++	NC	NC
CXCR4	BCR	NC	NC	NC
	TLR2	++	NC	NC

+ low expression

++ medium expression

+++ high expression

- slight downregulation

--- high downregulation

* NC = no change

their migration in GALT. The observation that PBL-1, 55D1 and 79E B cells all express CCR6 upon stimulation through TLR2 supports this idea.

It is possible that a group of early stage B cells expressing CCR6, like PBL-1 cells, enter GALT and follow the chemokine gradient to the FAE, where they are exposed to certain environmental and survival signals that lead them to migrate to the basolateral end of the follicle. In the basolateral end of the follicle, the cells undergo somatic hypermutation and Ig diversification (like 79E cells). Following a chemokine gradient and other environmental signals, the somatically diversified cells traffic back to the FAE and encounter bacterial molecules from the expanding population of commensals. These events may trigger signals that induce the expansion of B cells in GALT and the migration to the periphery.

Bacterial molecules and B cell migration

As discussed above, I successfully demonstrated *in vitro* that bacterial molecules can induce the activation of rabbit B cells. In addition to this, I demonstrated that stimulation through the pattern recognition receptor (PRR) TLR2 can induce the expression of certain chemokine receptors on the surface of B cells. However, these findings do not demonstrate that bacteria provide signals for B cell migration.

B. subtilis eps and *B. pumilus* molecules can stimulate the activation of B cells as evidenced by the expression of B7. On the other hand, PPRs like TLRs can recognize bacterial patterns. S. Jain (2013) demonstrated that the simultaneous signaling through

TLR2 and B7 results in the activation of resting B cells. Therefore, it was possible that eps and *B. pumilus* molecules could also stimulate the expression of chemokine receptors on activated B cells. To test this idea, I selected PBL-1 and 55D1 B cells. I reasoned that the majority of chemokine receptor expression or downregulation upon stimulation occurred in these two cell lines (Table 3), and not so much in 79E cell.

I observed that neither PBL-1, nor 55D1 B cells upregulated CCR6, CXCR5 and CCR7 when stimulated with bacterial molecules; and only CXCR4 was slightly up-regulated upon stimulation with *B. pumilus* molecules. It is possible that eps and/or *B. pumilus* molecules stimulate rabbit B cells through BCR only, or through other unidentified receptor(s) not involved in B cell migration. Although it was mentioned before that TLR2 could be the receptor for eps, this is not yet confirmed.

These experiments were performed once and their results are not sufficient to formulate a strong conclusion. The data provided by these experiments do not explain if the activation of B cells is linked to the expression of molecules involved in migration, such as chemokine receptors CCR6, CXCR5, CCR7 and CXCR4.

CONCLUSION

For many years the Knight Lab has been focusing on experiments designed to identify the bacterial molecule(s) that drives the activation and migration of B cells in the GALT of rabbits. In addition, our Lab has been trying to determine the role of intestinal bacteria in the mechanism of selection of V_HA B cells in rabbits.

The initial goal of this research was to determine the mechanism of selection of V_Hα B cells in rabbits. To test this idea, I isolated three species of interest, *B. pumilus*, *B. thuringiensis* and *P. acnes*, which were identified by ribosomal 16sRNA sequencing. During these experiments, I successfully identified a ~20kDa bacterial molecule from the isolated intestinal bacteria that immunoprecipitated with FvIg molecules. These data indicated that bacterial molecules can bind Ig, and supported the idea that in the FAE, bacteria provide B cells with signals that may lead to their activation.

However, over the course of my study I became interested in the mechanism by which bacterial molecules drive the activation and migration of B cells in the GALT of rabbits. While testing this question, I showed that bacterial molecules provide signals, through surface receptors (BCR and/or TLR2), that stimulate the activation of rabbit B cells. I also demonstrated that stimulation through TLR2 alters the expression of chemokine receptors on the surface of B cells.

These studies provide additional evidence in the quest to elucidate the mechanism by which interactions between the host and intestinal microbiota alter the B cell repertoire in an antigen independent manner.

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